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(57) Abstract

The present invention relates, in general, to the bioeffector molecule ceramide and, in particular, to methods of effecting intracellular accumulation of ceramide. The invention further relates to methods of selecting compounds that inhibit alkaline ceramidase and that can be used to treat diseases/disorders associated with cell hyperplasia or dedifferentiation.

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INHIBITOR OF CERAMIDASE

This is a continuation-in-part of Application No. 08/652,238, filed May 23, 1996, the entire contents of which is incorporated by reference.

This invention was made with Government support under Grant Nos. GM 43825 and DK 45067 awarded by the National Institutes of Health. The Government has certain rights in this invention.

TECHNICAL FIELD

The present invention relates, in general, to the bioeffector molecule ceramide and, in particular, to methods of effecting intracellular accumulation of ceramide. The invention further relates to methods of selecting compounds that inhibit alkaline ceramidase and that can be used to treat diseases/disorders associated with cell hyperplasia or dedifferentiation.

BACKGROUND

Ceramide is an important bioeffector lipid molecule (Hannun, J. Biol. Chem. 269:3125 (1994), Merrill, Jr., Nutr. Rev. 50:78 (1992), Kolesnick and Fuks, J. Exp. Med. 181:1949 (1995), Chao, Mol. Cell. Neurosci. 6:91 (1995), Liscovitch, Trends Biochem. Sci. 17:393 (1992)). The action of a number of

extracellular agents as well as stress stimuli, such as 1α,25-dihydroxyvitamin D_3, tumor necrosis factor α, interleukin-1ß, neurotrophins, the Fas ligand, dexamethasone, serum withdrawal, chemotherapeutic agents, and γ-irradiation, causes an elevation in the endogenous levels of ceramide (Hannun, J. Biol. Chem. 269:3125 (1994), Hannun and Obeid, Trends Biochem. Sci. 20:73 (1995), Ballou et al, J. Biol. Chem. 267:20044 (1992), Quintans et al, Biochem. Biophys. Res. Commun. 202:710 (1994), Dobrowsky et al, Science 265:1596 (1994), Yanaga and Watson, FEBS Lett. 314:297 (1992), Dressler and Kolesnick, Science 255:1715 (1992)). A role for endogenous ceramide in mediating, at least in part, the actions of these stimuli on cell differentiation, apoptosis, and growth suppression is supported by the ability of exogenous analogs of ceramide to induce these biologic responses in the respective cell types (Hannun, J. Biol. Chem. 269:3125 (1994), Okazaki et al, J. Biol. Chem. 265:15823 (1990), Bielawska et al, FEBS Lett. 307:211 (1992), Obeid et al, Science 259:1769 (1993), Laulederkind et al, J. Exp. Med. 182:599 (1995), Goldkorn et al, J. Biol. Chem. 266:16092 (1991)).

Additional evidence for a physiologic function of endogenous ceramide has come from studies examining the specificity of action of ceramide analogs. Thus, it has been shown with C_2-ceramide (D-*erythro*-*N*-acetylsphingosine) that this molecule demonstrates structural and stereospecific cellular

activities (Bielawska et al, J. Biol. Chem. 268:26226 (1993), Fishbein et al, J. Biol. Chem. 268:9255 (1993)). The most significant specificity was demonstrated with the lack of activity of C_2-dihydroceramide (Bielawska et al, J. Biol. Chem. 268:26226 (1993), Tepper et al, Proc. Natl. Acad. Sci. USA 92:8443 (1995)), which differs from C_2-ceramide by lacking the *trans* 4-5 double bond in the sphingoid base; otherwise, these two molecules display identical stereochemistry at the two chiral carbons (C-2 and C-3). Moreover, the cellular uptake and metabolism of these two compounds is nearly identical (Bielawska et al, J. Biol. Chem. 268:26226 (1993)), suggesting that the lack of activity of dihydroceramide is due to inability to interact with relevant intracellular targets. Indeed, C_2-ceramide but not C_2-dihydroceramide is able to activate ceramide-activated protein phosphatase (CAPP) *in vitro* (Fishbein et al, J. Biol. Chem. 268:9255 (1993), Dobrowsky et al, J. Biol. Chem. 268:15523 (1993)).

In addition, some of the cellular activities of ceramide have been mimicked by metabolic manipulation of endogenous ceramide levels. Thus, the addition of bacterial sphingomyelinase, which cleaves outer leaflet sphingomyelin and causes accumulation of membrane ceramide, has been shown to mimic at least some of the effects of exogenous cell permeable ceramides (Okazaki et al, J. Biol. Chem. 264:19076 (1989), Mathias et al, Science 259:519 (1993)).

Moreover, PDMP and related compounds, which inhibit cerbroside synthase (Abe et al, J. Biochem. (Tokyo) 111:191 (1992)) and also cause accumulation of ceramide, result in cellular activities shared with ceramide. These experimental approaches have lent further credence to a role for endogenous ceramide in cell regulation. They also point to potential versatility in regulation of ceramide levels. Indeed, ceramide occupies a central position in sphingolipid metabolism. Complex sphingolipids derive from ceramide through various enzymatic reactions that add various head groups to the 1-hydroxyl position (Hannun, J. Biol. Chem. 269:3125 (1994), Wiegandt in Glycolipids (Weigandt, ed) pp. 199-259, Elsevier, New York (1985), Merrill, Jr. and Jones, Biochim. Biophys. Acta 1044:1 (1990), Van Echten and Sandhoff J. Biol. Chem. 268:53412 (1993), Hakomori, Annu. Rev. Biochem. 50:733 (1981)). The breakdown of these sphingolipids through sequential metabolic reactions also results in the formation of ceramide. In turn, ceramide can be degraded further through the action of ceramidases resulting in the formation of sphingosine and free fatty acids (Hannun, J. Biol. Chem. 269:3125 (1994), Spence et al, Biochem. Cell Biol. 64:400 (19867), Slife et al, J. Biol. Chem. 264:10371 (1989)).

The activity of a number of synthetic ceramide analogs based on *N*-acylated phenylaminopropanols has been examined (Bielawska et al, J. Biol. Chem. 267:18493 (1992), USP 5,369,030). Two of these

analogs, D-*erythro*-MAPP (D-*erythro*-2-(N-myristolyamino)-1-phenyl-1-propanol) and L-*erythro*-MAPP (L-*erythro*-2-(N-myristolyamino)-1-phenyl-1-propanol), are of particular interest because they demonstrated enantiomeric selectivity of action. Examination of the absolute stereochemistry of these molecules (shown in Fig. 1) reveals that D-*erythro*-MAPP has an absolute configuration corresponding to L-*erythro*-ceramide, whereas L-*erythro*-MAPP has an absolute configuration corresponding to D-*erythro*-ceramide.

The present invention results, at least in part, from the demonstration that L-*e*-MAPP is a substrate for alkaline ceramidase whereas D-*e*-MAPP inhibits this enzyme and results in substantial elevation in intracellular levels of ceramide. This demonstration makes possible methods of selecting compounds that act as specific inhibitors of alkaline ceramidase, which compounds can be used to treat a variety of hyperproliferative diseases/disorders.

SUMMARY OF THE INVENTION

It is a general object of the invention to provide a method of selecting compounds that cause intracellular accumulation of ceramide.

It is a specific object of the invention to provide a method of identifying compounds that can be used to effect the selective inhibition of alkaline ceramidase.

It is a further object of the invention to provide a method of selecting compounds that can be used to treat hyperproliferative diseases/disorders.

The foregoing objects are met by the present invention.

In one embodiment, the present invention relates to a method of increasing intracellular levels of ceramide in a mammal. The method comprises administering to the mammal an agent that inhibits alkaline ceramidase in an amount sufficient to effect the inhibition, and monitoring the intracellular levels of ceramide.

In a further embodiment, the present invention relates to a method of treating a disease or disorder associated with cell hyperplasia. The method comprises administering to a mammal in need of such treatment an agent that inhibits alkaline ceramidase in an amount sufficient to effect the inhibition, and monitoring intracellular levels of ceramide in the mammal.

In yet another embodiment, the present invention relates to a method of testing a compound for its ability to inhibit mammalian alkaline ceramidase. The method comprises

i) contacting the compound with a sample comprising mammalian alkaline ceramidase, in the presence of ceramide, under conditions such that the alkaline ceramidase degrades ceramide to sphingosine in the absence of the compound, and

ii) determining the level of degradation of ceramide resulting from step (i) and comparing that level to a level of degradation obtained in the absence of the compound.

Further objects and advantages of the invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structures of D-*e*-ceramide, D-*e*-MAPP, and L-*e*-MAPP. The structure of *D-erythro*-ceramide is shown vertically-inverted to allow ease of comparison with the configuration of the polar head groups of D- and L-*e*-MAPP. Also shown are the Fisher projections for D-*e*-ceramide and L-*e*-MAPP. In absolute configuration, D-*e*-ceramide corresponds to L-*e*-MAPP whereas D-*e*-MAPP has the enantiomeric configuration.

Figure 2. Effects of D- and L-*e*-MAPP on cell growth. HL-60 cells were treated with 5 μM of either D-*e*-MAPP or L-*e*-MAPP and growth was monitored over the indicated time range (A). The effects of 5 μM D- and L-*e*-MAPP on cell cycle progression was determined at 48 h (B).

Figure 3. Effects of D- and L-*e*-MAPP on CAPP. The serine/threonine phosphatase activity of CAPP was determined using as substrate myelin basic protein

phosphorylated by protein kinase A. □, C_6-ceramide; ○, D-MAPP; Δ, L-MAPP.

Figure 4. Effects of D- and L-*e*-MAPP on endogenous levels of ceramide. HL-60 cells were treated with 5 μM D- or L-*e*-MAPP, and lipids were extracted for ceramide measurements at the indicated time points. A, the effects of D- and L-*e*-MAPP on total ceramide levels standardized to total levels of lipid phosphate. □, control; ●, D-MAPP; ▲, L-MAPP. B, TLC separation of diacylglycerol kinase products of synthetic *D-erythro*-C_{18}-ceramide, D-*erythro*-C_{18}-dihydroceramide, and ceramide from bovine brain sphingomyelin. C, relative changes in the levels of ceramide and dihydroceramide following the addition of D-*e*-MAPP to cells. Ceramide and dihydroceramide were quantitated as the respective phosphates.

Figure 5. Uptake and metabolism of D- and L-MAPP, HL-60 cells were treated with tritium-labeled D- and L-*e*-MAPP. A, back-extraction of D-*e*-MAPP taken up by cells. HL-60 cells were incubated with 5 μM tritium-labeled D-*e*-MAPP, and radioactivity associated with cells was determined before (no wash) or following the indicated number of washes. B, effect of duration of exposure of HL-60 cells to D-*e*-MAPP on growth suppression. HL-60 cells were treated with 5 μM D-*e*-MAPP which was back-extracted with 5 washes at the indicated time points. C, total uptake of D-*e*-MAPP and L-*e*-MAPP. HL-60 cells were

treated with the indicated concentrations of tritium-labeled D- and L-*e*-MAPP, and total radioactivity associated with the cell pellet was determined. ■, 5 μM D-MAPP; □, 30 μM D-MAPP; □, 5 μM L-MAPP; □, 30 μM L-MAPP; D, TLC separation of intact tritium-labeled *D*- and *L*-*e*-MAPP and their metabolites. Cells were treated with 5 μM D- or L-*e*-MAPP, and lipids were extracted at the 24-h time point and resolved on TLC. E, time dependence of metabolism of D- and L-*e*-MAPP. HL-60 cells were treated with 5 μM D- or L-*e*-MAPP. Intact compounds and their metabolites were resolved by TLC and radioactivity associated with each spot was determined by scintillation counting of the excised spots. Δ, D-MAPP; ●, L-MAPP; ○, L-MAPP (upper spot); ■, myristoyl-CoA.

Figure 6. Effects of D- and L-*e*-MAPP on alkaline and acid ceramidase activity *in vitro*. Ceramidase activity was determined from total homogenates of HL-60 cells. A, pH dependence of ceramidase activity from HL-60 cells. Ceramidase activity using tritium-labeled C_{16}-ceramide was measured *in vitro* using sodium acetate, HEPES, and CHES buffers over a pH range of 4.5 to 9.0. B, substrate preference of ceramidase *in vitro*. Tritium-labeled C_{16}-ceramide, D-*e*-MAPP, or L-*e*-MAPP were used as substrates in an *in vitro* ceramidase assay at 50 μM concentrations. Activity was determined by measuring release of the tritium-labeled acyl chains. C, inhibition of acid

ceramidase by D-*e*-MAPP and *N*-oleoylethanolamine. Ceramidase activity was evaluated *in vitro* at pH 4.5 using 50 μM C_{16} ceramide as a substrate in the presence of increasing concentrations of D-*e*-MAPP or *N*-oleoylethanolamine. Data are presented as mean activity normalized to control activity in samples treated with vehicle alone. D, inhibition of alkaline ceramidase by D-*e*-MAPP and *N*-oleoylethanolamine. Ceramidase activity was evaluated *in vitro* at pH 9.0 using 50 μM C_{16} ceramide as a substrate in the presence of increasing concentrations of D-*e*-MAPP (D-MAPP) or *N*-oleoylethanolamine (NOE). Data are presented as mean activity normalized to control activity in samples treated with vehicle alone.

Figure 7. Effects of fumonisin B1 on growth suppression by D-*e*-MAPP. HL-60 cells were treated with either vehicle or 3 μM D-MAPP in the presence of the indicated concentrations of fumonisin B1. Cell growth was determined at 48 h.

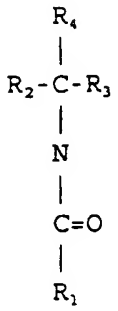
Figure 8. Interaction of D- and L-*e*-MAPP in cells. A, the effects of D-e-MAPP on cellular metabolism of L-*e*-MAPP. HL-60 cells were treated with 3 μM tritium-labeled L-e-MAPP with or without 3 μM D-*e*-MAPP. Metabolism of L-MAPP was determined. B, concentration dependence of inhibition of L-*e*-MAPP metabolism in cells. The upper spot on TLC reflects

a major metabolite of L-MAPP that probably represents triacylglycerol.

Figure 9. Scheme of ceramide metabolism and known inhibitors. Ceramide can be interconverted to sphingomyelin, cerebroside, or sphingosine through the action of at least 6 different enzymatic activities: 1) sphingomyelin synthase; 2) sphingomyelinase; 3) cerebroside synthase; 4) cerebrosidase; 5) ceramidase; 6) ceramide synthase.

DETAILED DESCRIPTION OF THE INVENTION

The present invention results, at least in part, from the observation that D-*e*-MAPP functions as an inhibitor of alkaline ceramidase. The biological consequences of this inhibition mimic those associated with exogenous ceramides. These observations make possible the regulation of endogenous ceramide levels and the identification of compounds that can be used to selectively inhibit alkaline ceramidase activity and thereby inhibit cell growth or induce cell differentiation. It is expected that compounds suitable for use in the invention include, generally, amides of hydrophobic molecules having the stereochemistry of D-*e*-MAPP, eg, molecules of the formula



wherein R_1, R_2, R_3 and R_4 are such that of the molecule is hydrophobic and no two of R_1, R_2, R_3 and R_4 are hydrogen.

USP 5,369,030 relates to the use of N-acyl-phenylaminoalcohol analogs to induce cellular differentiation and to alter cell phenotype. USP 5,369,030 further relates to the use of such analogs to treat diseases/disorders characterized by cell hyperproliferation. The compounds disclosed in USP 5,369,030 and their methods of use (for example, in treating malignant (eg cancer, leukemia and lymphoma), premalignant (eg myelodysplasia) or benign (eg lymphoma, proliferative, benign tumors and psoriasis) diseases/disorders) are incorporated herein by reference.

One compound disclosed in USP 5,369,030, D-*e*-MAPP, is shown in the Examples that follow to be capable of effecting concentration- and time-dependent suppression of cell growth, accompanied by an arrest in the G_0/G_1 phase of the cell cycle. The data presented in the Examples demonstrate that these effects result from the selective inhibition by D-*e*-MAPP of alkaline

ceramidase activity. This demonstration makes it possible to screen compounds for their ability to increase endogenous ceramide levels by selectively inhibiting alkaline ceramidase activity and to distinguish between compounds that increase ceramide levels by inhibiting degradation of ceramide to sphingosine and those that do not result in such inhibition.

In a broad sense, the present invention relates to a method of treating a disease or disorder associated with cell dedifferentiation or hyperproliferation (for example, treating malignant (eg cancer, including breast cancer, prostate cancer, lung cancer, leukemia and lymphoma), premalignant (eg myelodysplasia) or benign (eg lymphoma, proliferative, benign tumors and psoriasis) diseases/disorders). The method comprises administering to an individual in need of such treatment an agent that selectively inhibits alkaline ceramidase, in an amount sufficient to effect the inhibition. Suitable agents include N-acyl-phenylaminoalcohols of USP 5,369,030, specifically, compounds disclosed therein having an absolute stereochemical configuration opposite that D-*erythro*-ceramide.

The above-indicated compounds can be prepared as described in USP 5,369,030, and formulated into pharmaceutical compositions as described therein. Administration protocols can be used as described in USP 5,369,030 to effect the indicated treatments.

Compounds, in addition to those described above, can be selected for their suitability for use in treating diseases and disorders associated with cell hyperplasia or dedifferentiation by screening test compounds for their ability to inhibit alkaline ceramidase. Such screens constitute a further embodiment of the invention. In this embodiment, purified or partially purified preparations of ceramidase (either naturally occurring or recombinantly produced) can be used (examples of suitable preparations are described in the Examples that follow). A reaction mixture comprising the enzyme, ceramide and the test compound can be incubated, for example, at a pH in the range of 6.5 to 10 (preferably about 9.0), at about 37°C for about 1 hour. The reaction can be monitored, for example, by determining the extent of ceramide degradation or sphingosine or fatty acid production. In the Examples that follow, ceramide labeled with a detectable label (eg radioactive (eg tritiated) ceramide) is used as substrate and the reaction monitored by separating the labeled products (eg radiolabeled-fatty acid). Various other techniques for monitoring can be used, however, including various other detectable labels (eg fluorescent labels).

In accordance with this screening approach, the ceramidase activity is determined at various concentrations of the test compound. Those compounds that result in about a 50% inhibition of enzyme

activity, or greater, at a concentration in the range of 1 nM to 50 μM are preferred.

In addition to a system of the type described above, the present invention also related to screens wherein a known alkaline ceramidase inhibitor is incubated with a test compound, a ceramidase preparation and ceramide (under conditions, for example, as described above). The ability of the test compound to shift the dose response curve of the known inhibitor to the left is indicative of a compound that inhibits alkaline ceramidase and thus that is useful in treating cell hyperproliferative disorders/diseases. Compounds that enhance alkaline ceramidase inhibitory activity can be further tested for stability, toxicity, etc, using standard protocols.

Compounds selected for their ability to inhibit alkaline ceramidase can be formulated into pharmaceutical compositions and used therapeutically as described above. Subjects susceptible to therapy involving the use of the present compounds can be identified by isolating a biological sample from the subject and determining whether ceramidase present in that sample can be inhibited (for example, under conditions such as those described above) by the selected inhibitor.

It will be appreciated that the preferred subject of the invention is a human, however, veterinary uses are also contemplated.

The demonstration that compounds of the D-e-MAPP-type inhibit alkaline ceramidase makes possible protocols suitable for purifying the enzyme. For example, potent inhibitors can be used to affinity purify ceramidase, for example, from crude cellular preparations using art recognized affinity purification techniques.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLES

The following experimental details relate to the Examples that follow.

Proliferation and cell cycle studies. HL-60 human myelocytic leukemia cells (obtained from the American Type Culture Collection) were grown in RPMI 1640 medium containing 10% fetal calf serum at 37°C in 5% CO_2 incubator. For proliferation and cell cycle studies, cells were resuspended at a density of 2 X 10^5 cells/ml in serum-free media containing insulin (5 mg/liter) and transferrin (5 mg/liter) and sodium selenite (5 μg/liter) for 2-4 h. Cells were then treated with the indicated compounds. Ethanol concentration was always maintained at less than 0.1%. Cell proliferation was determined by counting cells using a hemacytometer, cell viability was evaluated by trypan blue dye exclusion, and cell

cycle analysis was determined by propidium iodide flow cytometry as described (Chao et al, J. Biol. Chem. 267:23459 (1992)).

Synthesis and labeling of D-e-MAPP and L-e-MAPP. D- and L-MAPP were prepared by acylation of D- and L-*erythro*-2-amino-1-phenyl-1-propanol with myristoyl chloride as described (Bielawska et al, J. Biol. Chem. 267:18493 (1992)). 2-(*N*-[^{3}H]myristolamino)-1-phenyl-1-propanols, D-[^{3}H]MAPP, and L-[^{3}H]MAPP were prepared following the general procedure for MAPP synthesis using [^{3}H]myristic acid converted to [^{3}H]myristoyl chloride or *N*-succinimidyl-[^{3}H]myristoanate.

Ceramide and diacylglycerol levels. Ceramide and diacylglycerol levels were measured on total liquid extracts using the diacylglycerol kinase assay (Jayadev et al, J. Biol. Chem. 270:2047 (1995)).

Synthesis of 2-(N-[^{3}H-palmitylamino)-sphingosine ([^{3}H]C_{16}-ceramide). Synthesis was performed by acylation of (2S,3R)-sphingosine with [^{3}H]palmitic acid converted to [^{3}H]palmitoyl chloride or *N*-succinimidyl-[^{3}H]palmitoanate (Jayadev et al, J. Biol. Chem. 270:2047 (1995)). A crude product was purified via the flash chromatography method (EM-Science silica gel; 40-65 μm) using a methylene chloride/methanol system with increasing polarity from 100:0 to 98:4 and were crystallized from ethanol. The purity of the obtained compound was assessed by TLC analysis using Merck pre-coated Silica Gel 60 F-254 plates and methylene

chloride/methanol, 93:7, or chloroform/methanol, 4:1, solvent systems to develop plates. TLC spot detection was by iodine vapor and 5% potassium parmanganate in 1 N potassium hydroxide. Structure of nonradioactive amides was verified by proton-NMR (^{1}H NMR), mass spectroscopy (MS), and CD spectra. Specific activity of the obtained *N*-[^{3}H]acyl derivatives was ~3-4 X 10^4 dpm/nmol, and the purity was close to 100%.

Uptake and metabolism studies. HL-60 cells were grown as described and treated with ^{3}H-labeled compounds at concentrations ranging from 0 to 10μM. At the indicated time points, cell pellets were separated from media and ^{3}H radioactivity was counted.

Metabolism studies. Lipids from cells treated with ^{3}H-labeled compounds were extracted (Bligh & Dyer method), dried, resuspended in chloroform/methnol (10:1, v/v), and subjected to separation by TLC in chloroform/methanol (4:1, v/v). Spots containing radioactive compounds were scraped and radioactivity was measured using a scintillation counter.

Measurement of ceramidase activity in HL-60 cells. Ceramidase activity in HL-60 cells was measured by a modification of the method of Gatt and Yavin (Methods Enzymol. 14:139 (1969)). Cells were disrupted by sonication in 0.25 M sucrose, 1 mM EDTA, and centrifuged at 10,000 X g for 10 min after which the supernatant was centrifuged at 100,000 xg for 60 min. Ten μl of 1 mM ceramide substrate ([^{3}H]C_{16}-ceramide) was mixed with 100 μl of Triton X-100

(0.1%) in chloroform/methanol, 2:1, and 100 μl of 0.2% sodium cholate in chloroform/methanol, 2:1, after which the solvent was evaporated under N_2 at 70°C. To this mixture, 30 μl of water was added and the tubes were heated for 5s in an 80°C water bath and then put on ice. Fifty μl of the appropriate buffer depending on desired pH was added followed by 20 μl of 50 mM $MgCl_2$ and 100 μl of cell extract. In experiments using inhibitors, the desired inhibitor was added in ethanol either with the substrate or directly to the final solution. The reaction mixture was incubated for 1 h at 37°C. The ^{3}H-fatty acid products of ceramidase were separated by the addition of 2 ml of Dole's solution (isopropyl alcohol, heptane, NaOH), 1.2 ml of heptane, and 1 ml of water. After vortexing and centrifugation, the upper phase was discarded and the lower phase was washed twice with heptane and the upper phase discarded each time. Finally, 1 ml of 1 N H_2SO_4, and 2 ml of heptane were added, and after vortexing and centrifugation the upper phase was transferred for counting by liquid scintillation.

Glucocerebrosidase and glucocerebroside synthase assays. *In vitro* assays for gluccerebrosidase and for cerebroside synthase were performed as described (Abe et al, J. Biochem. (Tokyo) 11:191 (1992)).

CAPP. The activity of CAPP was determined as described previously (Dobrowsky and Hannun, J. Biol. Chem. 267:5048 (1992)) using myelin basic protein (phosphorylated by protein kinase A) as a substrate.

EXAMPLE I

Cellular Activition of D- and L-Erythro-MAPP

In structure-function analysis of ceramide-mediated growth suppression using amides of phenylaminoalcohols, D- and L-*erythro*-MAPP were employed. In these studies, HL-60 cells were treated with 5 μM of either D-*e*-MAPP or L-*e*-MAPP, and growth was determined at the indicated time points (Fig. 2A). D-*erythro*-MAPP produced a time dependent suppression of growth that was predominantly characterized by a cytostatic effect on proliferation. On the other hand, L-*erythro*-MAPP was largely inactive (Fig. 2A).

In order to evaluate if D-*erythro*-MAPP modulated cell cycle progression, HL-60 cells were treated with 5 μM D-*e*-MAPP, and cell analysis was performed at 48 h using propidium iodide flow cytometry. These studies showed that *D-erythro*-MAPP produced a significant increase in the population of cells in the G_0/G_1 phase of the cell cycle (from 60 to 73%) with a corresponding drop in the population of cells in S (from 30 to 24%) and G_2/M (from 10 to 3%) phases of the cell cycle (Fig. 2B). On the other hand, 5 μM L-*e*-MAPP was largely without effect (Fig. 2B). These studies show that a predominant effect of D-*e*-MAPP is the induction of a G_0/G_1 arrest in cell cycle progression resulting in growth suppression.

EXAMPLE II

Effects on CAPP

Since D-*e*-MAPP mimicked the cellular activities of ceramide, it was determined whether D-*e*-MAPP mimicked the *in vitro* activity of ceramides. *In vitro* ceramides of varying *N*-linked chain lengths activate a serine/threonine protein phosphatase (CAPP), with a specificity matching the specificity of the cellular activities of ceramides (Wolff et al, J. Biol. Chem. 269:19605 (1994)). CAPP appears to belong to the heterotrimeric subfamily of the PP2A family of protein phosphatases (Dobrowsky et al, J. Biol. Chem. 268:15523 (1993)). Addition of C_6-ceramide to CAPP *in vitro* resulted in a concentration-dependent stimulation of activity (Fig. 8). On the other hand, neither D-*e*-MAPP nor L-*e*-MAPP activated CAPP *in vitro* (Fig. 3), raising the possibility that D-*e*-MAPP may not function as a direct analog of ceramide.

EXAMPLE III

Effects of D- and L-MAPP on Endogenous Levels of Ceramide

Since D-*erythro*-MAPP, the bioactive enantiomer, corresponds in absolute configuration to L-*erythro*-ceramide (the unnatural enantiomer of ceramide)

(Fig. 1), and since it lacks *in vitro* activity with CAPP, it was determined whether D-*e*-MAPP mimicked ceramide biology by modulation endogenous ceramide levels. Therefore, HL-60 cells were treated with 5 μM D-*e*-MAPP, ethanol vehicle or L-*e*-MAPP for 0-24 h. At the indicated time points (Fig. 4A), total lipids were extracted and endogenous ceramide levels were measured by the *Escherichia coli* diacylglycerol kinase assay. In control cells, there were no changes in endogenous ceramide levels, and in cells treated with L-*e*-MAPP there were very modest changes (~20%) in ceramide levels; especially at the early time points (Fig. 4A). However, the addition of D-*e*-MAPP resulted in a time dependent accumulation of endogenous levels of ceramide reaching approximately 3-fold of baseline by the 24-h time point. These results demonstrate that, as compared to L-*e*-MAPP, D-*e*-MAPP selectively regulates endogenous ceramide levels.

The results from the diacylglycerol kinase assay revealed that D-*e*-MAPP selectively modulated the levels mostly of one of two major species of ceramide phosphate (the product of ceramide transformation by the diacylglycerol kinase assay) as detected by TLC (Fig. 4B). The upper spot of ceramide phosphate co-migrated with synthetic *D-erythro*-C_{18} dihydroceramide phosphate whereas the lower spot co-migrated with D-*erythro*-C_{18} ceramide phosphate. Fig. 4C shows that the addition of D-*e*-MAPP resulted in selective enhancement in the levels of the lower

spot of ceramide phosphate. Based on this co-migration, the results suggest that D-*e*-MAPP causes a selective increase in ceramide but not dihydroceramide levels. It should be noted, however, that the structure of these species of ceramide phosphate has not been determined conclusively.

EXAMPLE IV

Uptake and Metabolism of D- and L-MAPP

Studies were conducted examining the uptake and metabolism of D- and L-MAPP. In the first set of studies, the effect of duration of exposure of HL-60 cells to D-*e*-MAPP on the bioactivity of the molecule was determined. D-*e*-MAPP added to cells could be washed off by repeated sedimentation and washing of cells. Thus, after five cycles of washing, less than 10% of D-MAPP remained associated with the cell pellet compared to unwashed cells (Fig. 5A). The continuous exposure of HL-60 cells to D-MAPP resulted in dramatic growth inhibition (Fig. 5A) as compared to vehicle-treated cells. Exposure of HL-60 cells to D-MAPP for only 15 min did not affect cell growth, whereas exposure for progressively longer intervals resulted in increased growth suppression (Fig. 5B). These results show that the ability of D-*e*-MAPP to inhibit growth requires prolonged exposure of cells to this compound and suggest that the effects of D-*e*-MAPP require continuous interactions with

intracellular targets (such as ceramide-metabolizing enzymes).

Next, the uptake of tritium-labeled D- and L-*e*-MAPP was examined. To this end, D- and L-*e*-MAPP labeled in the N-myristoyl group were synthesized and used for uptake and metabolism studies. The addition of 5 or 30 μM of either D- or L-*e*-MAPP resulted in a dose- and time-dependent increase in uptake of the molecules (Fig. 5C). At 24 hr, approximately 20% of D-e-MAPP (5 μM) and 26% of L-*e*-MAPP (5 μM) were taken up by cells.

The partitioning of the label taken up by cells into the lipid organic phase was evaluated next. It was found that the majority of the label in D-*e*-MAPP or L-*e*-MAPP remained associated with the organic phase over a 0-72 h duration at concentrations ranging from 5 to 30 μM.

Next, the metabolism of D- and L-*e*-MAPP was evaluated. To this end, organic lipids were extracted from cells treated with 5 μM D- or L-e-MAPP at the indicated time points (Fig. 5D), and lipids were resolved on TLC. D-*e*-MAPP showed little metabolism and remained intact throughout the duration of the experiment (Fig. 5, D and E). On the other hand, L-e-MAPP underwent significant metabolism in a time-dependent fashion such that by 24 h, more than 70% of the L-*e*-MAPP taken up by cells was metabolized (Fig. 5, D and E). Two major breakdown products of L-*e*-MAPP were resolved by TLC. One metabolite co-migrated with myristoyl-CoA and not

with other neutral or polar lipids. The other metabolite (upper spot in Fig. 5D) comigrated with triacylglycerol. These results are of 2-fold significance. First, they suggest that the major reason for the lack of activity of L-*e*-MAPP may be due to its relatively rapid metabolism. Second, they show that L-*e*-MAPP is a substrate for endogenous ceramidases that are capable of releasing the *N*-linked myristate. On the other hand, D-*e*-MAPP does not appear to be a substrate for this enzyme.

EXAMPLE V

Effects of D- and L-e-MAPP on Ceramide-Metabolizing Enzymes

In order to evaluate the interactions of D- and L-*e*-MAPP with ceramidase, *in vitro* studies we conducted using cytosolic and membrane fractions from HL-60 cells. Initially, ceramidase activity was examined for pH dependence using tritium-labeled C_{16}-ceramide as substrate. Ceramidase activity was found to exist predominantly in the membrane fraction at two separate pH optima of 4.5 and 9.0 (Fig. 6A). The alkaline ceramidase activity was very similar to that described in fibroblasts and cerebellar tissue (Sugita et al, Biochim. Biophys. Acta 398:125 (1975), Wertz and Downing, FEBS Lett. 268:110 (1990)). In initial studies, the ability of this ceramidase to utilize D-e- and L-*e*-MAPP as substrates was

investigated. D-*e*-MAPP was not substrate for the alkaline ceramidase (Fig. 6B). In contrast, L-*e*-MAPP was readily hydrolyzed by ceramidase to a level equivalent to that seen with C_{16}-ceramide as a substrate (Fig. 6B). These studies corroborate the cellular metabolism studies showing that L-*e*-MAPP is a substrate for ceramidase *in vitro* and in cells.

Next, the ability of D- and L-*e*-MAPP to interfere with hydrolysis of C_{16}-ceramide by ceramidase at pH 4.5 and 9.0 was evaluated. Inhibition of ceramidase activity by D-*e*-MAPP was compared to that by *N*-oleoylethanolamine, a putative inhibitor of acid ceramidase (Sugita et al, Biochim. Biophys. Acta 398:125 (1975)). *N*-Oleoylethanolamine was found to be a better inhibitor of acid ceramidase (IC_{50} approximately 500 μM) than D-*e*-MAPP (Fig. 6C). The effects of *N*-oleoylethanolamine are consistent with the previously reported K_i of 700 μM (Sugita et al, Biochim. Biophys. Acta 398:125 (1975)). D-*e*-MAPP demonstrated much greater inhibition of alkaline ceramidase with an IC_{50} of 1-5 μM (Fig. 6C). D-*e*-MAPP displayed an apparent *in vitro* K_i for alkaline ceramidase of 2-13 X 10^6 μM. At a final concentration of 5 μM, D-*e*-MAPP inhibited alkaline ceramidase *in vitro* by approximately 60% whereas *N*-oleoylethanolamine displayed less than 10% inhibition of alkaline ceramidase *in vitro* at final concentrations up to 50 μM (Fig. 6D). In contrast, D-*e*-MAPP was a relatively poor inhibitor of acid ceramidase with less than 10% inhibition of acid

ceramidase at 50 μM (Fig. 6C). These results demonstrate that D-*e*-MAPP is a much superior inhibitor of alkaline ceramides than *N*-oleoylethanolamine and displays specificity for *in vitro* inhibition of alkaline ceramidase over acid ceramidase. These results are consistent with the use of 0.5 mM *N*-oleoylethanolamine to inhibit mitogenesis (Coroneos et al, J. Biol. Chem. 270:23305 (1995)) which is 100-fold higher than the concentration of D-*e*-MAPP used to inhibit cell growth in Fig. 2.

In addition to ceramidase, inhibition of cerebroside synthase and sphingomyelin synthase or stimulation of sphingomyelinase or cerebrosidase could result in elevations in endogenous ceramide levels. Therefore, the effects of D- and L-*e*-MAPP were examined on these enzyme activities *in vitro* and in cells. Neither D- nor L-*e*-MAPP caused inhibiton of cerebroside synthase activity (Table I). As a control, PMMP, a previously established inhibitor of cerebroside synthase, induced significant inhibition of this enzymatic activity (Table I). Also, D- and L-*e*-MAPP did not activate or modulate the activity of ß-glucosidase. In addition, neither D- nor L-*e*-MAPP modulated the endogenous levels of sphingomyelin arguing against an effect of either of these molecules on sphingomyelinase or sphingomyelin synthase. *In vitro*, neither D- nor L-*e*-MAPP modulated the activity of neutral or acidic sphingomyelinases. Finally, fumonisin B1, an

inhibitor of ceramide synthase (45), did not inhibit the effects of D-*e*-MAPP on growth (Fig. 7).

TABLE I

Glucosylceramide synthase activity (nmol/h/mg protein)	
Control	1.009 ± 0.0424
D-*e*-MAPP (20 μM)	0.946 ± 0.0265
L-*e*-MAPP (20 μM)	1.009 ± 0.0383
PMMP (20 μM)	0.208 ± 0.008

EXAMPLE VI

Effects of D-e-MAPP on Ceramidase Activity in Cells

Since D-*e*-MAPP inhibits ceramidase in cells, then it should also protect L-*e*-MAPP from metabolic degradation. Therefore, the effects of D-*e*-MAPP on metabolism of L-*e*-MAPP were examined. HL-60 cells were treated with tritium-labeled L-e-MAPP (3 μM), and the metabolism of L-e-MAPP was evaluated in the presence and absence of 3 μM D-*e*-MAPP. The addition of D-*e*-MAPP prevented the breakdown of L-e-MAPP (Fig. 8A). The effects of D-*e*-MAPP on metabolism of L-*e*-MAPP were dose dependent with most of the inhibition occurring at levels of D-*e*-MAPP of 1-3 μM (Fig. 8B). Therefore, these studies demonstrate that D-*e*-MAPP inhibits the metabolism of L-*e*-MAPP in cells through inhibition of the involved ceramidase.

* * *

All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of increasing intracellular levels of ceramide in a mammal comprising administering to said mammal an agent that inhibits alkaline ceramidase in an amount sufficient to effect said inhibition, and monitoring said intracellular levels of ceramide.

2. The method according to claim 1 wherein agent is a N-acyl-phenylaminoalcohol.

3. The method according to claim 2 wherein agent is D-*erythro*-2-(N-myristolyamino)-1-phenyl-1-propanol (D-*e*-MAPP).

4. A method of treating a disease or disorder associated with cell hyperplasia or dedifferentiation comprising administering to a mammal in need of such treatment an agent that inhibits alkaline ceramidase in an amount sufficient to effect said inhibition, and monitoring intracellular levels of ceramide in said mammal.

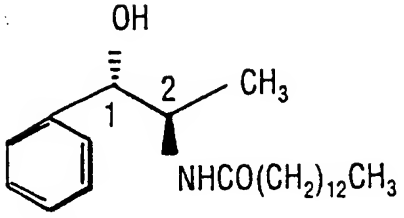
5. The method according to claim 4 wherein agent is a N-acyl-phenylaminoalcohol.

6. The method according to claim 4 wherein agent is D-*erythro*-2-(N-myristolyamino)-1-phenyl-1-propanol (D-*e*-MAPP).

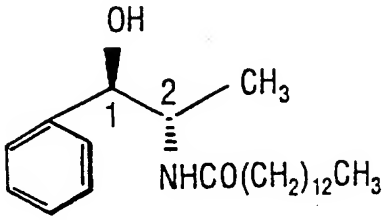
7. A method of testing a compound for its ability to inhibit mammalian alkaline ceramidase comprising

i) contacting said compound with a sample comprising mammalian alkaline ceramidase, in the presence of ceramide, under conditions such that said alkaline ceramidase degrades ceramide to sphingosine in the absence of said compound, and

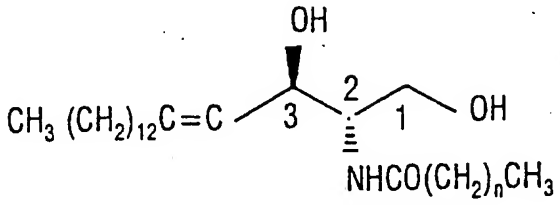
ii) determining the level of degradation of said ceramide and comparing that level to a level of degradation obtained in the absence of said compound.



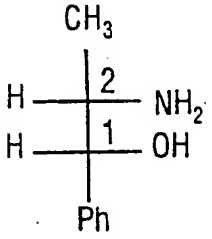
(IS, 2R) D-e-MAPP



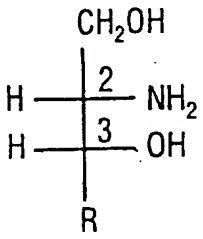
(IR,2S) L-e-MAPP



(2S, 3R, 4E) D-e-Ceramide



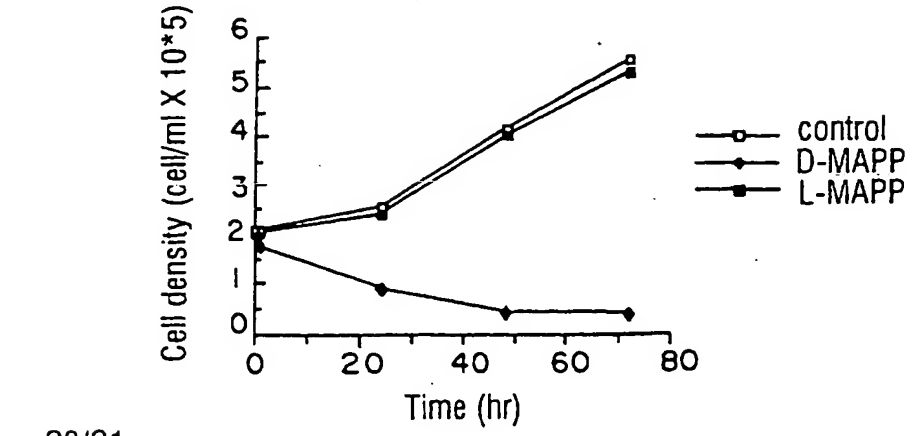
L-e-MAPP



D-e-Ceramide

Fig. 1

Fig. 2A


Cell density (cell/ml X 10*5)
6
5
4
3
2
1
0
0
20
40
60
80
Time (hr)
control
D-MAPP
L-MAPP

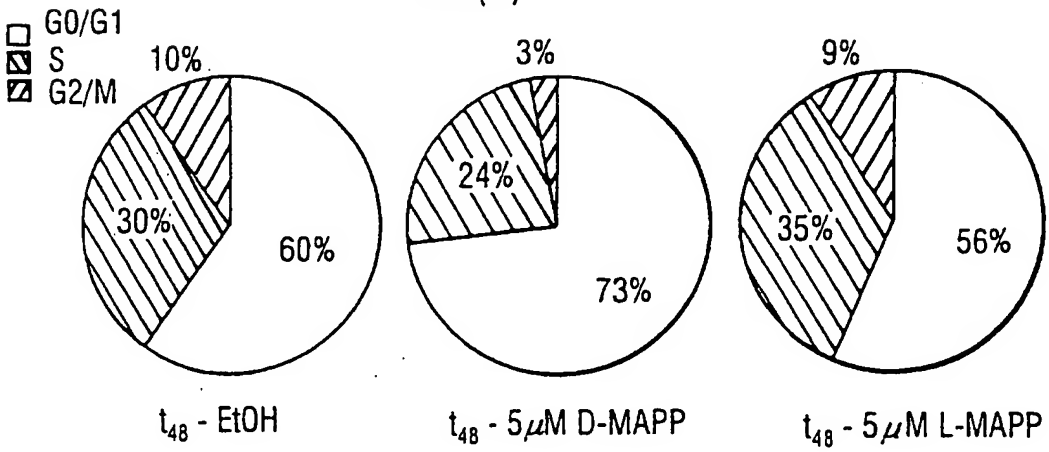

G0/G1
S
G2/M
10%
30%
60%
3%
24%
73%
9%
35%
56%
t_{48} - EtOH
t_{48} - 5μM D-MAPP
t_{48} - 5μM L-MAPP

Fig. 2B

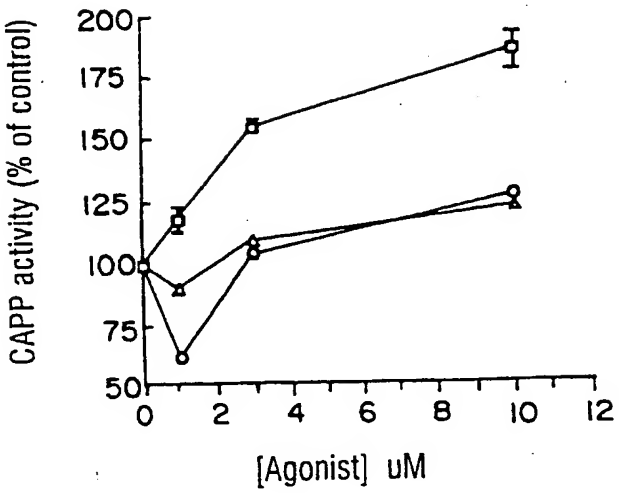

CAPP activity (% of control)
200
175
150
125
100
75
50
0
2
4
6
8
10
12
[Agonist] uM

Fig. 3

Fig. 4A

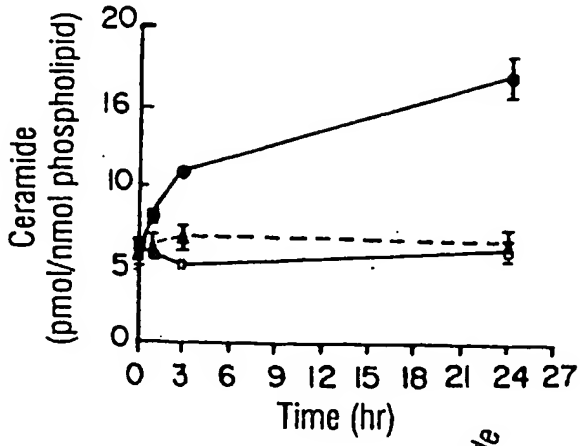
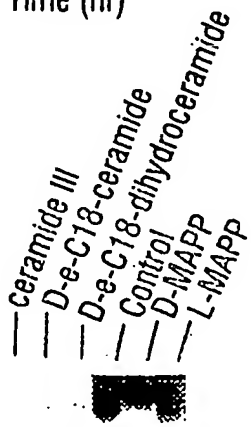


Fig. 4B



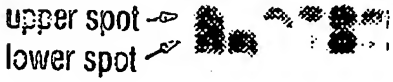
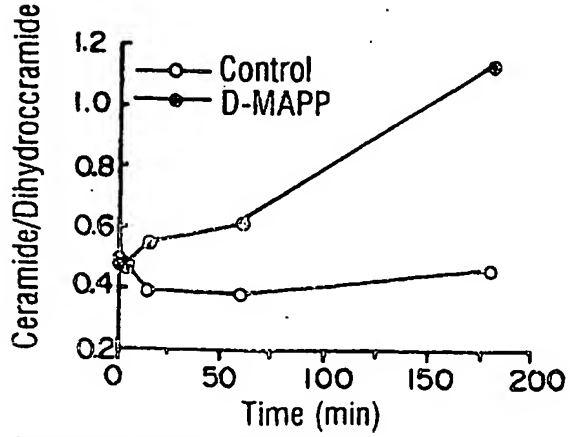


Fig. 4C



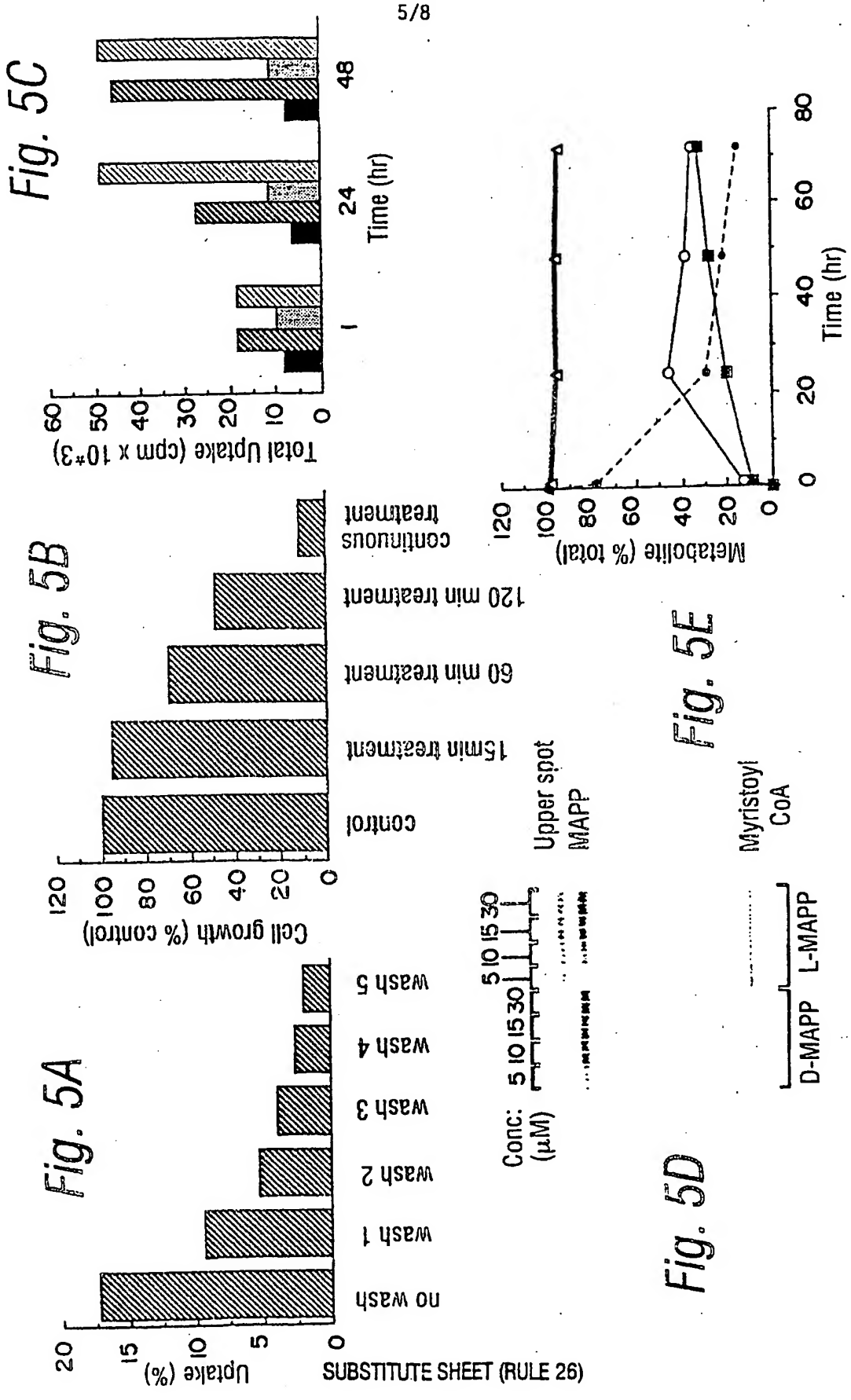


Fig. 5A
Uptake (%)
0
5
10
15
20
no wash
wash 1
wash 2
wash 3
wash 4
wash 5
Conc: (μM)
5 10 15 30
5 10 15 30
Upper spot
MAPP
Fig. 5B
Cell growth (% control)
0
20
40
60
80
100
120
control
15min treatment
60 min treatment
120 min treatment
continuous treatment
Fig. 5C
Total Uptake (cpm x 10^3)
0
10
20
30
40
50
60
1
24
48
Time (hr)
Fig. 5D
Myristoyl CoA
D-MAPP
L-MAPP
Fig. 5E
Metabolite (% total)
0
20
40
60
80
100
120
0
20
40
60
80
Time (hr)

Fig. 6A

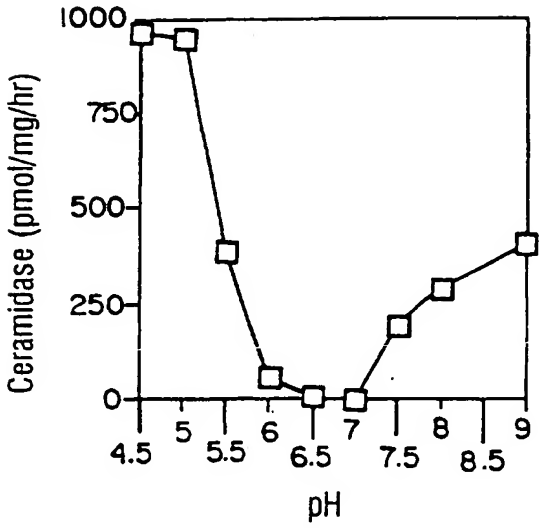
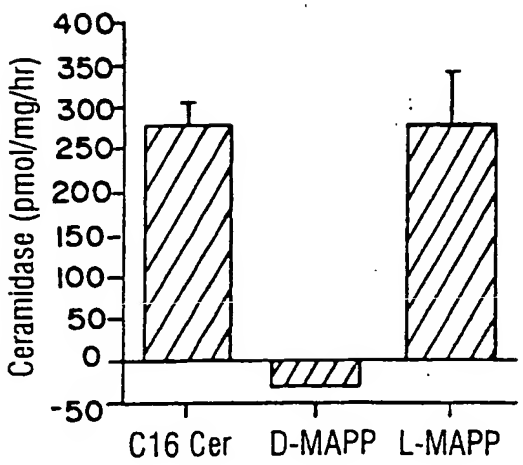


Fig. 6B



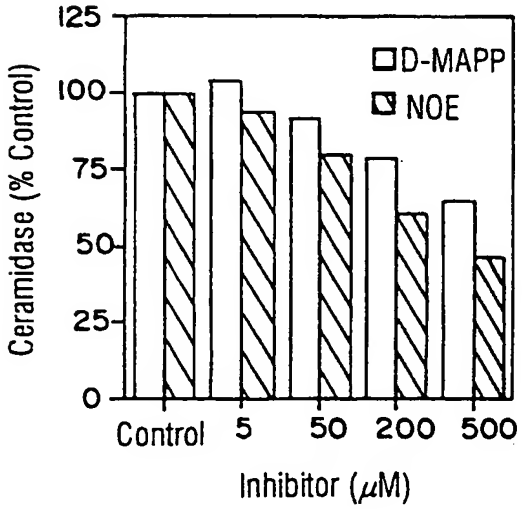


Fig. 6C

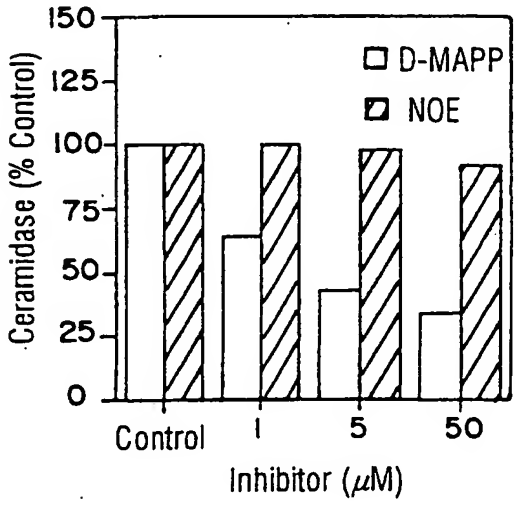
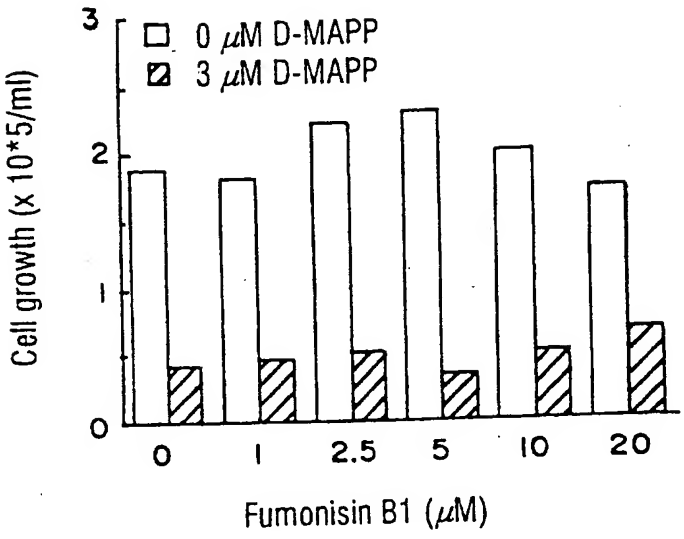


Fig. 6D

Fig. 7


0 μM D-MAPP
3 μM D-MAPP
Cell growth (x 10*5/ml)
3
2
1
0
0
1
2.5
5
10
20
Fumonisin B1 (μM)

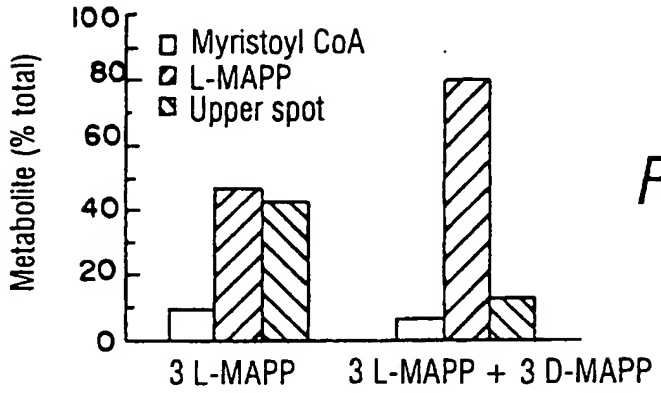


Fig. 8A

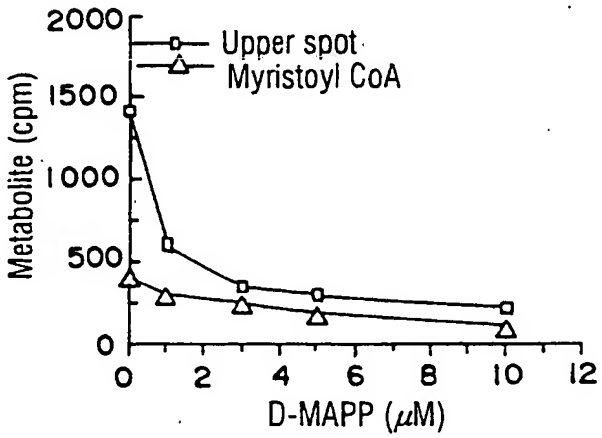
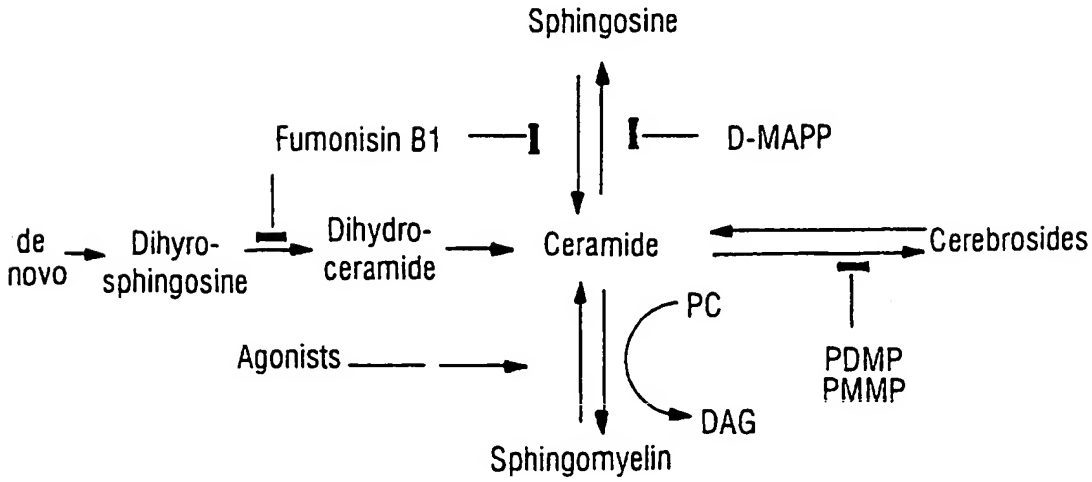


Fig. 8B

Fig. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17769

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/16; C12N 5/00.
US CL :435/240.1, 240.2; 514/625, 629.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 240.2; 514/625, 629.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,369,030 A (HANNUN et al.) 29 November 1994, column 2, line 25 to column 3, line 6.	1-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 MARCH 1997	Date of mailing of the international search report 04 APR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RICHARD L. RAYMOND Telephone No. (703) 308-1235

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With amended claims.

(54) Title: AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE

(57) Abstract

Novel amino ceramide-like compounds are provided which inhibit glucosyl ceramide (GlcCer) formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of glycosphingolipids. The compounds of the present invention have improved GlcCer synthase inhibition activity and are therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered glycosphingolipid levels.

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AMINO CERAMIDE - LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE

SPONSORSHIP

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FIELD OF THE INVENTION

The present invention relates generally to ceramide-like compounds and, more particularly, to ceramide-like compounds containing a tertiary amine group and their use in therapeutic methods.

BACKGROUND OF THE INVENTION

Hundreds of glycosphingolipids (GSLs) are derived from glucosylceramide (GlcCer), which is enzymatically formed from ceramide and UDP-glucose. The enzyme involved in GlcCer formation is UDP-glucose:N-acylsphingosine glucosyltransferase (GlcCer synthase). The rate of GlcCer formation under physiological conditions may depend on the tissue level of UDP-glucose, which in turn depends on the level of glucose in a particular tissue (Zador, I.Z. et al., "A Role for Glycosphingolipid Accumulation in the Renal Hypertrophy of Streptozotocin-Induced Diabetes Mellitus," *J. Clin. Invest.* 91:797-803 (1993)). *In vitro* assays based on endogenous ceramide yield lower synthetic rates than mixtures containing added ceramide, suggesting that tissue levels of ceramide are also normally rate-limiting (Brenkert, A. et al., "Synthesis of Galactosyl Ceramide and Glucosyl Ceramide by Rat Brain: Assay Procedures and Changes with Age," *Brain Res.* 36:183-193 (1972)).

It has been found that the level of GSLs controls a variety of cell functions, such as growth, differentiation, adhesion between cells or between cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor cells. In addition, the GlcCer precursor, ceramide, may cause differentiation or inhibition of cell growth (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters* 307:211-214 (1992)) and be involved in the functioning of vitamin D_3, tumor necrosis factor-α, interleukins, and apoptosis (programmed cell death). The sphingols (sphingoid bases), precursors of ceramide, and products of ceramide catabolism, have also been shown to influence many cell systems, possibly by inhibiting protein kinase C (PKC).

It is likely that all the GSLs undergo catabolic hydrolysis, so any blockage in the GlcCer synthase should ultimately lead to depletion of the GSLs and profound changes in the functioning of a cell or organism. An inhibitor of GlcCer synthase, PDMP (1R-phenyl-2R-decanoylamino-3-morpholino-1-propanol), previously identified as the D-threo isomer (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987)), has been found to produce a variety of chemical and physiological changes in cells and animals (Radin, N.S. et al., "Use of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol (PDMP), an Inhibitor of Glucosylceramide Synthesis," *In NeuroProtocols, A Companion to Methods in Neurosciences*, S. K. Fisher et al., Ed., (Academic Press, San Diego) 3:145-155 (1993) and Radin, N.S. et al., "Metabolic Effects of Inhibiting Glucosylceramide Synthesis with PDMP and Other Substances," *In Advances in Lipid Research; Sphingolipids in Signaling, Part B.*, R.M. Bell et al., Ed. (Academic Press, San Diego) 28:183-213 (1993)). Particularly interesting is the compound's ability to cure mice of cancer induced by Ehrlich ascites carcinoma cells (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.* 38:23-30 (1987)), to produce accumulation of sphingosine and *N,N*-dimethylsphingosine (Felding-Habermann, B. et al., "A Ceramide Analog Inhibits T Cell Proliferative Response Through Inhibition of Glycosphingolipid Synthesis and Enhancement of *N,N*-Dimethylsphingosine Synthesis," *Biochemistry* 29:6314-6322 (1990)), and to slow cell growth (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). Compounds with longer chain fatty acyl groups have been found to be substantially more effective (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.* 111:191-196 (1992)).

The importance of GSL metabolism is underscored by the seriousness of disorders resulting from defects in GSL metabolizing enzymes. For example, Tay-Sachs, Gaucher's, and Fabry's diseases, resulting from enzymatic defects in the GSL degradative pathway and the accumulation of GSL in the patient, all have severe clinical manifestations. Another example of the importance of GSL function is seen in a mechanism by which blood cells, whose surfaces contain selectins, can, under certain conditions, bind to GSLs in the blood vessel walls and produce acute, life-thr atening inflammation (Alon, R. et al., "Glycolipid Ligands for Selectins

Support Leukocyte Tethering & Rolling Under Physiologic Flow Conditions." *J. Immunol.*, 154:5356-5366 (1995)).

At present there is only one treatment available for patients with Gaucher disease, wherein the normal enzyme which has been isolated from normal human tissues or cultured cells is administered to the patient. As with any drug isolated from human material, great care is needed to prevent contamination with a virus or other dangerous substances. Treatment for an individual patient will be extremely expensive, costing hundreds of thousands, or even millions of dollars, over a patient's lifetime. It would thus be desirable to provide a treatment which includes administration of a compound that is readily available and/or producible from common materials by simple reactions.

Possibly of even greater clinical relevance is the role of glucolipids in cancer. For example, it has been found that certain GSLs occur only in tumors; certain GSLs occur at abnormally high concentrations in tumors; certain GSLs, added to tumor cells in culture media, exert marked stimulatory or inhibitory actions on tumor growth; antibodies to certain GSLs inhibit the growth of tumors; the GSLs that are shed by tumors into the surrounding extracellular fluid inhibit the body's normal immunodefense system; the composition of a tumor's GSLs changes as the tumors become increasingly malignant; and, in certain kinds of cancer, the level of a GSL circulating in the blood gives useful information regarding the patient's response to treatment. Because of the significant impact GSLs have on several biochemical processes, there remains a need for compounds having improved GlcCer synthase inhibition activity.

It would thus be desirable to provide compounds which inhibit GlcCer synthase activity. It would also be desirable to provide compounds which inhibit GlcCer synthase activity, thereby lowering the level of GSLs and increasing GSL precursor levels, *e.g.* increasing the levels of ceramide and sphingols. It would further be desirable to provide compounds which inhibit GlcCer synthase activity and lower the level of GSLs without also increasing ceramide levels. It would also be desirable to provide compounds and therapeutic methods to treat conditions and diseases associated with altered GSL levels and/or GSL precursor levels.

SUMMARY OF THE INVENTION

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibition activity and are

therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels, as well as GSL precursor levels. For example, the compounds of the present invention may be useful in methods involving cancer growth and metastasis, the growth of normal tissues, the ability of pathogenic microorganisms to bind to normal cells, the binding between similar cells, the binding of toxins to human cells, and the ability of cancer cells to block the normal process of immunological cytotoxic attack.

Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

Figure 1 is a graph showing the growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors;

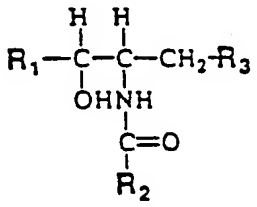
Figure 2 is a graph showing the protein content of MDCK cells cultured for 24 hr in medium containing different concentrations of the separated erythro- and threo-isomers of a preferred compound of the present invention; and

Figure 3 is a graph showing [^{3}H]thymidine incorporation into the DNA of MDCK cells treated with a preferred compound of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibitory activity and are therefore highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels.

The compounds of the present invention generally have the following formula:



wherein

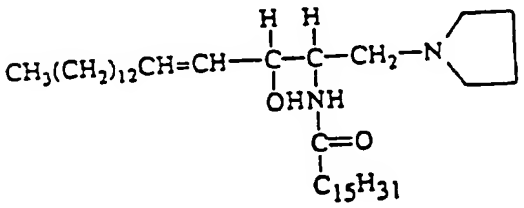
R_1 is a phenyl group, preferably a substituted phenyl group such as p-methoxy, cyclohexyl or other acyclic group, t-butyl or other branched aliphatic group, or a long alkyl or alkenyl chain, preferably 7 to 15 carbons long with a double bond next to the kernel of the structure. The aliphatic chain can have a hydroxyl group near the two asymetric centers, corresponding to phytosphingosine.

R_2 is an alkyl residue of a fatty acid, 10 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (*e.g.*, a hydroxyl group).

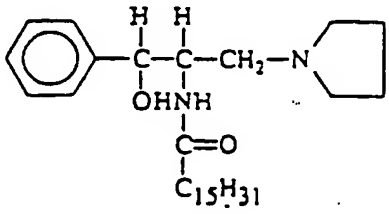
R_3 is a tertiary amine, preferably a cyclic amine such as pyrrolidine, azetidine, morpholine or piperidine, in which the nitrogen atom is attached to the kernel (*i.e.*, a tertiary amine).

All four structural isomers of the compounds are contemplated within the present invention and may be used either singly or in combination (*i.e.*, DL-threo or DL-erythro).

The preferred aliphatic compound of the present invention is D-threo-1-pyrrolidino-1-deoxyceramide, identified as IV-231B herein and also referred to as PD. The preferred aromatic compound of the present invention is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, identified as BML-119 herein and also referred to as P4. The structures of the preferred compounds are as follows:



PD



P4

By increasing the acyl chain length of PDMP from 10 to 16 carbon atoms, the efficacy of the compounds of the present invention as GlcCer synthase inhibitors is greatly enhanced. The use of a less polar cyclic amine, especially a pyrrolidine instead of a morpholine ring, also increases the efficacy of the compounds. In addition, replacement of the phenyl ring by a chain corresponding to sphingosine yields a strongly inhibitory material. By using a chiral synthetic route, it was discovered that the isomers active against GlcCer synthase had the R,R-(D-threo)-configuration. However, strong inhibition of the growth of human

cancer cells *in plastico* was produced by both the threo and erythro racemic compounds, showing involvement of an additional factor beyond simple depletion of cell glycosphingolipids by blockage of GlcCer synthesis. The growth arresting effects could be correlated with increases in cellular ceramide and diglyceride levels.

Surprisingly, the aliphatic pyrrolidino compound of the present invention (identified as IV-231B), was strongly inhibitory toward the GlcCer synthase and produced almost complete depletion of glycolipids, but did not inhibit growth or cause an accumulation of ceramide. Attempts were made to determine if the differences in growth effects could be attributed to the influence of the inhibitors on related enzymes (ceramide and sphingomyelin synthase and ceramidase and sphingomyelinase). While some stimulation or inhibition of enzyme activity was noted, particularly at high inhibitor concentrations (50 μM), these findings did not explain the differing effects of the different inhibitors.

By slowing the synthesis of GlcCer, the compounds of the present invention lower the levels of all the GlcCer-derived GSLs due to the GSL hydrolases which normally destroy them. While the body will continue to make the more complex GSLs from available GlcCer, the rate of synthesis will slow down as the level of GlcCer diminishes. The rate of lowering depends on the normal rate of destruction of each GSL. These rates however, are relatively rapid in animals and cultured cells.

At higher dosages, many of the compounds of the present invention produce an elevation in the level of ceramide. Presumably this occurs because cells continue to make ceramide despite their inability to utilize it for GlcCer synthesis. Ceramide is also normally converted to sphingomyelin, but this process does not seem to be able to handle the excess ceramide. It has been unexpectedly found however, that an additional process is also involved, since even those isomers that are inert against GlcCer synthase also produce an elevation in ceramide levels. Moreover, the blockage of GlcCer synthase can occur at low inhibitor dosages, yet ceramide accumulation is not produced. The preferred aliphatic compound of the present invention, D-threo-1-pyrrolidino-1-deoxyceramide (PD), does not produce ceramide accumulation at all, despite almost complete blockage of GlcCer synthesis.

This distinction between the aromatic and the aliphatic compounds of the present invention is important because ceramide has recently b en proposed to cause cell death (apoptosis) by some still unknown mechanism. At lower dose levels, the aromatic compounds of the present invention cause GSL disapperance

with only small accumulation of ceramide and inhibition of cell growth. Higher dosages cause much more ceramide deposition and very slow cell growth or cell death.

In one embodiment of the present invention, methods of treating patients suffering from inborn genetic errors in the metabolism of GlcCer and its normal anabolic products (lactosylceramide and the more complex GSLs) are provided. The presently known disorders in this category include Gaucher, Fabry, Tay-Sachs, Sandhoff, and GM1 gangliosidosis. The genetic errors lie in the patient's inability to synthesize a hydrolytic enzyme having normal efficiency. Their inefficient hydrolase allows the GSL to gradually accumulate to a toxic degree, debilitating or killing the victim. The compounds of the present invention slow the formation of GSLs, thus allowing the defective hydrolase to gradually "catch up" and restore the concentrations of GSLs to their normal levels and thus the compounds may be administered to treat such patients.

With respect to Gaucher disease, it has been calculated that much of the patient's accumulated GlcCer in liver and spleen arises from the blood cells, which are ultimately destroyed in these organs after they have reached the end of their life span. The actual fraction, lipid derived from blood cells versus lipid formed in the liver and spleen cells, is actually quite uncertain, but the external source must be important. Therefore it is necessary for the compounds of the present invention to deplete the blood cells as they are formed or (in the case of white blood cells) while they still circulate in the blood. Judging from toxicity tests, the white cells continue to function adequately despite their loss of GSLs. Although the toxicity studies were not of a long enough duration to produce many new red cells with low GSL content, it is possible that circulating red cells also undergo turnover (continual loss plus replacement) of GSLs.

In an alternative embodiment of the present invention, for the treatment of disorders involving cell growth and division, high dosages of the compounds of the present invention are administered but only for a relatively short time. These disorders include cancer, collagen vascular diseases, atherosclerosis, and the renal hypertrophy of diabetic patients. Accumulation or changes in the cellular levels of GSLs have been implicated in these disorders and blocking GSL biosynthesis would allow the normal restorative mechanisms of the body to resolve the imbalance.

With atherosclerosis, it has been shown that arterial epithelial cells grow faster in the presence of a GlcCer product (lactosylceramide). Oxidized serum

lipoprotein, a material that normally circulates in the blood, stimulates the formation of plaques and lactosylceramide in the inner lining of blood vessels. Treatment with the compounds of the present invention would inhibit this mitogenic effect.

In an additional embodiment of the present invention, patients suffering from infections may be treated with the compounds of the present invention. Many types of pathogenic bacteria have to bind to specific GSLs before they can induce their toxic effects. As shown in Svensson, M. et al., "Epithelial Glucosphingolipid Expression as a Determinant of Bacterial Adherence and Cytokine Production," *Infect. and Immun.* 62:4404-4410 (1994), expressly incorporated by reference, PDMP treatment reduces the adherence of *E. coli* to mammalian cells. Several viruses, such as influenza type A, also must bind to a GSL. Several bacterial toxins, such as the verotoxins, cannot themselves act without first binding to a GSL. Thus, by lowering the level of GSLs, the degree of infection may be ameliorated. In addition, when a patient is already infected to a recognizable, diagnosable degree, the compounds of the present invention may slow the further development of the infection by eliminating the binding sites that remain free.

It has been shown that tumors produce substances, namely gangliosides, a family of GSLs, that prevent the host *i.e.*, patient, from generating antibodies against the tumor. By blocking the tumor's ability to secrete these substances, antibodies against the tumor can be produced. Thus, by administering the GlcCer synthase inhibitors of the present invention to the patient, the tumors will become depleted of their GSLs and the body's normal immunological defenses will come into action and destroy the tumor. This technique was described in Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.* 38:23-30(1987), expressly incorporated by reference. The compounds of the present invention and in particular the aliphatic compounds require much lower doses than those previously described. This is particularly important because the lower dose may reduce certain side effects. Moreover, because the aliphatic compounds of the present invention do not produce ceramide accumulation, they are less toxic. In addition, 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), may act via two pathways, GSL depletion and ceramide accumulation.

In an alternative embodiment, a vaccine-like preparation is provided. Here, cancer cells are removed from the patient (preferably as completely as possible), and the cells are grown in culture in order to obtain a large number of the cancer cells. The cells are then exposed to the inhibitor for a time sufficient to deplete the

cells of their GLSs (generally 1 to 5 days) and are reinjected into the patient. These reinjected cells act like antigens and are destroyed by the patient's immunodefense system. The remaining cancer cells (which could not be physically removed) will also be attacked by the patient's antibodies. In a preferred embodiment, the patient's circulating gangliosides in the plasma are removed by plasmapheresis, since the circulating gangliosides would tend to block the immunodefense system.

It is believed that tumors are particularly dependent on GSL synthesis for maintenance of their growth (Hakomori, S. "New Directions in Cancer Therapy Based on Aberrant Expression of Glycosphingolipids: Anti-adhesion and Ortho-Signaling Therapy," *Cancer Cells* 3:461-470 (1991)). Accumulation of ceramide in treated tumors also slows their growth or kills them. Tumors also generate large amounts of GSLs and secrete them into the patient's body, thereby preventing the host's normal response by immunoprotective cells, which should generate antibodies against or otherwise destroy tumor cells (*e.g.*, tumors are weakly antigenic). It has also been shown that GSL depletion blocks the metastasis of tumor cells (Inokuchi, J. et al., "Inhibition of Experimental Metastasis of Murine Lewis Long Carcinoma by an Inhibitor of Glucosylceramide Synthase and its Possible Mechanism of Action," *Cancer Res.* 50:6731-6737 (1990). Tumor angiogenesis (*e.g.*, the production of blood capillaries) is strongly influenced by GSLs (Ziche, M. et al., "Angiogenesis Can Be Stimulated or Repressed in *In Vivo* by a Change in GM3:GD3 Ganglioside Ratio," *Lab. Invest.* 67:711-715 (1992)). Depleting the tumor of its GSLs should block the tumors from generating the new blood vessels they need for growth.

A further important characteristic of the compounds of the present invention is their unique ability to block the growth of multidrug resistant ("MDR") tumor cells even at much lower dosages. This was demonstrated with PDMP by Rosenwald, A.G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, on Lysosomes in Cultured Cells," *J. Lipid Res.* 35:1232 (1994), expressly incorporated by reference. Tumor cells that survive an initial series of therapeutic treatments often reappear some years later with new properties - they are now resistant to a second treatment schedule, even with different drugs. This change has been attributed to the appearance in the tumor of large amounts of a specific MDR protein (P-glycoprotein). It has been suggested that protein kinase C (PKC) may be involved in the action or formation of P-glycoprotein (Blobe, G.C. et al., "Regulation of PKC and Its Role in Cancer Biology," *Cancer Metastasis Rev.* 13:411-431 (1994)). However decreases in PKC have other important effects, particularly slowing of

growth. It is known that PDMP does lower the cellular content of PKC (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)) but it is not clear why it so effectively blocks growth of MDR cells (Rosenwald, A.G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, On Lysosomes in Cultured Cells," *J. Lipid Res.* 35:1232 (1994)). A recent report showed that several lipoidal amines that block MDR action also lower the level of the enzyme acid sphingomyelinase (Jaffrezou,J. et al., "Inhibition of Lysosomal Acid Sphingomyelinase by Agents which Reverse Multidrug Resistance," *Biochim. Biophys. Acta* 1266:1-8 (1995)). One of these agents was also found to increase the cellular content of sphingosine 5-fold, an effect seen with PDMP as well. One agent, chlorpromazine, behaves like the compounds of the present invention, in its ability to lower tissue levels of GlcCer (Hospattankar, A.V. et al., "Changes in Liver Lipids After Administration of 2-Decanoylamino-3-Morpholinopropiophenone and Chlorpromazine," *Lipids* 17:538-543 (1982)).

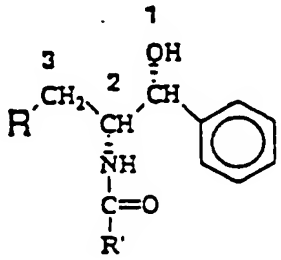
It will be appreciated by those skilled in the art that the compounds of the present invention can be employed in a wide variety of pharmaceutical forms; the compound can be employed neat or admixed with a pharmaceutically acceptable carrier or other excipients or additives. Generally speaking, the compound will be administered orally or intravenously. It will be appreciated that therapeutically acceptable salts of the compounds of the present invention may also be employed. The selection of dosage, rate/frequency and means of administration is well within the skill of the artisan and may be left to the judgment of the treating physician or attending veterinarian. The method of the present invention may be employed alone or in conjunction with other therapeutic regimens. It will also be appreciated that the compounds of the present invention are also useful as a research tool *e.g.*, to further investigate GSL metabolism.

The following Specific Example further describes the compounds and methods of the present invention.

SPECIFIC EXAMPLE

The following formulas set forth preferred aromatic and aliphatic compounds:

FORMULA I



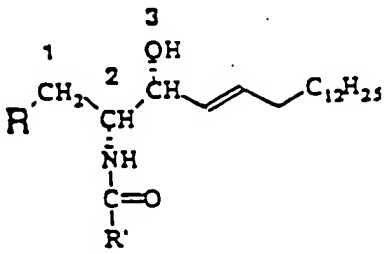
identified as (1R,2R)-1-phenyl-2-acylamino-3-cyclic amino-1-propanoi, and referred to herein as the "aromatic inhibitors," wherein

The phenyl group can be a substituted phenyl group (such as *p*-methoxyphenyl).

R' is an alkyl residue of a fatty acid, 10 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (*e.g.*, a hydroxyl group).

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.

FORMULA II



identified as (2R,3R)-2-palmitoyl-sphingosyl amine or 1-cyclic amino-1-deoxyceramide or 1-cyclic amino-2-hexadecanoylamino-3-hydroxy-octadec-4,5-ene, and referred to herein as the "aliphatic inhibitors," wherein

R' is an alkyl residue of a fatty acid, 10 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (*e.g.*, a hydroxyl group).

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.

The long alkyl chain shown in Formula II can be 8 to 18 carbon atoms long, with or without a double bond near the asymertric carbon atom (carbon 3). Hydroxyl groups can, with advantage, be substituted along the aliphatic chain, particularly on carbon 4 (as in the naturally occurring sphingol, phytosphingosine). The long chain can also be replaced by other aliphatic groups, such at t-butyl or cyclopentyl.

The aromatic inhibitors (see Formula I and Table 1) were synthesized by the Mannich reaction from 2-*N*-acylaminoacetophenone, paraformaldehyde, and a secondary amine as previously described (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987) and Vunnam, R.R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids* 26:265-278 (1980)). For those syntheses in which phenyl-substituted starting materials were used, the methyl group in the acetophenone structure was brominated and converted to the primary amine. Bromination of p-methoxyacetophenone was performed in methanol. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, MO. Miscellaneous reagents were from Sigma Chemical Co. and the sphingolipids used as substrates or standards were prepared by methods known in the art. The reactions produce a mixture of four isomers, due to the presence of two asymmetric centers.

The aliphatic inhibitors (See Formula II and Table 2) were synthesized from the corresponding 3-*t*-butyldimethylsilyl-protected sphingols, prepared by enantioselective aldol condensation (Evans, D.A. et al., "Stereoselective Aldol Condensations Via Boron Enolates," *J. Am. Chem. Soc.* 103:3099-3111 (1981) and Abdel-Magid, A. et al., Metal-Assisted Aldol Condensation of Chiral A-Halogenated Imide Enolates: A Stereocontrolled Chiral Epoxide Synthesis," *J. Am. Chem. Soc.* 108:4595-4602 (1986)) using a modification of the procedure of Nicolaou et al. (Nicolaou, K.C. et al., "A Practical and Enantioselective Synthesis of Glycosphingolipids and Related Compounds. Total Synthesis of Globotriaosylceramide (Gb_3)," *J. Am. Chem. Soc.* 110:7910-7912 (1988)). Each protected sphingol was first converted to the corresponding primary triflate ester, then reacted with a cyclic amine. Subs quent *N*-acylation and desilylation led to the

final products in good overall yield (Carson, K.G. et al., "Studies on Morpholinosphingolipids: Potent Inhibitors of Glucosylceramide Synthase," *Tetrahedron Lett.* 35:2659-2662 (1994)). The compounds can be called 1-morpholino-(or pyrrolidino)-1-deoxyceramides.

Labeled ceramide, decanoyl sphingosine, was prepared by reaction of the acid chloride and sphingosine (Kopaczyk, K. C. et al., "*In Vivo* Conversions of Cerebroside and Ceramide in Rat Brain," *J. Lipid Res.* 6:140-145 (1965)) and NBD-SM (12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-sphingosylphosphorylcholine) was from Molecular Probes, Inc., Eugene, OR.

Methods

TLC of the amines was carried out with HPTLC plates (E. Merck silica gel 60) and C-M-HOAc 90:10:10 (solvent A) or 85:15:10 (solvent B) or C-M-conc. ammonium hydroxide 30:10:1 (solvent C). The bands were stained with iodine or with Coomassie Brilliant Blue R-250 (Nakamura, K. et al., "Coomassie Brilliant Blue Staining of Lipids on Thin-Layer Plates," *Anal. Biochem.* 142:406-41 (1984)) and, in the latter case, quantified with a Bio-Rad Model 620 videodensitometer operated with reflected white light. The faster band of each PDMP analog, previously identified as the erythro form, corresponds to the 1S,2R and 1R,2S stereoisomers, and the slower band, previously identified as the threo form, corresponds to the 1R,2R and 1S,2S stereoisomers.

TLC of the cell lipids was run with C-M-W 24:7:1 (solvent D) or 60:35:8 (solvent E).

Growth of cell lines. Comparisons of different inhibitors with regard to suppression of human cancer cell growth were made by the University of Michigan Cancer Center in vitro Drug Evaluation Core Laboratory. MCF-7 breast carcinoma cells, HT-29 colon adenocarcinoma cells, H-460 lung large cell carcinoma cells, and 9L brain gliosarcoma cells were grown in RPMI 1640 medium with 5% fetal bovine serum, 2 mM glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 0.1 mg/ml of neomycin. UMSCC-10A head and neck squamous carcinoma cells were grown in minimal essential medium with Earle salts and the same supplements. Medium components were from Sigma Chemical Co. Cells were plated in 96-well microtiter plates (1000 cells/well for H-460 and 9L cells, and 2000 cells/well for the other lines), and the test compounds were added 1 day later. The stock inhibitor solutions, 2 mM in 2 mM BSA, were diluted with different amounts of additional 2

mM BSA, then each solution was diluted 500-fold with growth medium to obtain the final concentrations indicated in the Figures and Tables.

Five days after plating the H-460 and 9L cells, or 6 days for the other lines, cell growth was evaluated by staining the adhering cells with sulforhodamine B and measuring the absorbance at 520 nm (Skehan, P. et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," *J. Natl. Cancer Inst.* 82:1107-1112 (1990)). The absorbance of the treated cultures is reported as percent of that of control cultures, to provide an estimate of the fraction of the cells that survived, or of inhibition of growth rate.

For the experiments with labeled thymidine, each 8.5 cm dish contained 500,000 Madin-Darby canine kidney (MDCK) cells in 8 ml of Dulbecco modified essential supplemented medium. The cells were incubated at 37°C in 5% CO_2 for 24 h, then incubated another 24 h with medium containing the inhibitor-BSA complex. The control cells were also incubated in the presence of BSA. The cells were washed with phosphate/saline and trichloroacetic acid, then scraped off the dishes, dissolved in alkali, and analyzed for protein and DNA incorporated tritium. [Methyl-^{3}H]thymidine (10 μCi) was added 4 h prior to harvesting.

Assay of sphingolipid enzymes. The inhibitors were evaluated for their effectiveness against the GlcCer synthase of MDCK cell homogenates by incubation in a thermostatted ultrasonic bath (Radin N.S. et al., "Ultrasonic Baths as Substitutes for Shaking Incubator Baths," *Enzyme* 45:67-70 (1991)) with octanoyl sphingosine and uridinediphospho[^{3}H]glucose (Shukla, G.S. et al., "Glucosylceramide Synthase of Mouse Kidney: Further Characterization and Improved Assay Method," *Arch. Biochem. Biophys.* 283:372-378 (1990)). The lipoidal substrate (85 μg) was added in liposomes made from 0.57 mg dioleoylphosphatidylcholine and 0.1 mg of Na sulfatide. Confluent cells were washed, then homogenized with a micro-tip sonicator at 0°C for 3 x 30 sec; ~0.2 mg of protein was used in each assay tube. In the case of the aromatic inhibitors, the test compound was simply evaporated to dryness from solution in the incubation tube. This method of adding the inhibitor was found to give the same results as addition as a part of the substrate liposomes. The aliphatic inhibitors, which appeared to be less soluble in water, were added as part of the substrate liposomes.

Acid and neutral ceramidases were assayed under conditions like those above, but the medium contained 110 μM [1-^{14}C]d canoyl sphingosine (10^5 cpm) in 340 μM dioleoylphosphatidylcholine liposomes and 0.34 mg of MDCK cellular protein

homogenate. The acid enzyme was incubated in 32.5 mM citrate-Na^+ (pH 4.5) and the neutral enzyme buffer was 40 mM Tris-Cl^- (pH 7.1 at 37°C). After 60 min in the ultrasonic bath, 3 ml of C-M 2:1, carrier decanoic acid, and 0.6 ml of 0.9% saline were added and the lipids in the lower layer were separated by TLC with C-HOAc 9:1. The liberated decanoic acid was scraped off the glass plate and counted.

Ceramide synthase was assayed with 1 μM [3-^{3}H]sphingosine (70,000 cpm, repurified by column chromatography), 0.2 mM stearoyl-CoA, 0.5 mM dithiothreitol, and ~300 μg of MDCK homogenate protein in 25 mM phosphate-K^+ buffer, pH 7.4, in a total volume of 0.2 ml. The incubation (for 30 min) and TLC were carried out as above and the ceramide band was counted.

Sphingomyelin synthase was evaluated with 44 μM [^{4}C]decanoyl sphingosine (10^5 cpm) dispersed with 136 μM dioleoyllecithin as in the ceramide synthase assay, and 5 mM EDTA and 50 mM Hepes-Na^+ pH 7.5, in a total volume of 0.5 ml. MDCK homogenate was centrifuged at 600 X g briefly, then at 100,000 X g for 1 h, and the pellet was suspended in water and sonicated with a dipping probe. A portion of this suspension containing 300 μg of protein was used. Incubation was at 37°C for 30 min, after which the lipids were treated as above, using C-M-W 60:35:8 for the isolation of the labeled decanoyl SM.

Acid and neutral SMase assays were based on the procedures of Gatt et al. (Gatt, S. et al., "Assay of Enzymes of Lipid Metabolism With Colored and Fluorescent Derivatives of Natural Lipids," *Meth. Enzymol.* 72:351-375 (1981)), using liposomes containing NBD-SM dispersed like the labeled ceramide (10 μM substrate and 30 μM lecithin). The assay medium for the neutral enzyme also contained 50 mM Tris-Cl^- (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.29 mg of MDCK cell protein in a total volume of 0.25 ml. Incubation was at 37°C for 30 min in the ultrasonic bath, then the fluorescent product, NBD-ceramide, was isolated by partitioning the assay mixture with 0.45 ml 2-propanol, 1.5 ml heptane, and 0.2 ml water. After centrifugation, a trace of contaminating NBD-SM was removed from 0.9 ml of the upper layer by washing with 0.35 ml water. The upper layer was analyzed with a fluorometer (460 nm excitation, 515 nm emission).

Acid SMase was assayed with the same liposomes in 0.2 ml of assay mixture containing 125 mM NaOAc (pH 5.0) and 61 μg of cell protein, with 60 min of incubation at 37°C. The resultant ceramide was determined as above.

Results

Table 1 lists the aromatic compounds (see Formula I) synthesized and their migration rates on silica gel TLC plates. Separation of the threo- and erythro-steroisomers by TLC was generally very good, except for BML-120, -121, and -122 in the acidic solvent. In the basic solvent BML-119 and BML-122 yielded poorly resolved double bands. BML-112 was unexpectedly fast-running, especially when compared with BML-120; both are presumably dihydrochlorides.

TABLE 1
Structures of the Aromatic Inhibitors

BML Number or Name	R Group	Phenyl Substituent	TLC hR_f Value[a]
PDMP[b]	morpholino		34(47)
PPMP	morpholino		(53)
112	N-phenylpiperazino		56
113	morpholino	*p*-fluoro	25
114	diethylamino		25
115	piperidino (pentamethyleneimino)		29
116	hexamethyleneimino		34
117[b]	morpholino	*p*-fluoro	41
118	piperidino	*p*-fluoro	26
119	pyrrolidino (tetramethyleneimino)		20-70(44)
120	1-methylpiperazino		7-62
121	3-dimethylaminopiperidino		1-30
122	N-methylethanolamino		6-71
123	azetidino (trimethyleneimino)		12
124	amino		15
125	morpholino	*p*-methoxy	37
126	pyrrolidino	*p*-methoxy	(50)

[a] Only the relative R_f value of the faster-moving band is shown. The first value was obtained with solvent A, the second with solvent C, and the numbers in parentheses, with solvent B. In the case of BML-117, -125, and -126, a 20-cm high TLC plate was used to improve the seperation.
[b] The fatty acid chain suggested by the R' group is decanoyl, not palmitoyl.

Table 2 describes four aliphatic inhibitors (see Formula II), which can be considered to be ceramide analogs in which the C-1 hydroxyl group is replaced by a cyclic amine. It should be noted that the carbon frameworks of compounds in Tables 1 and 2 are numbered differently (see Formulas I and II), thus affecting comparisons of stereochemical configurations. The threo- and erythro-isomers separated very poorly on TLC plates. Like the aromatic inhibitors, however, the morpholine compounds ran faster than the pyrrolidine compounds. The latter are presumably more strongly adsorbed by the silica gel because they are more basic.

TABLE 2
Characterization of the Sphingosyl Inhibitors

Number	R Group	Sphingol Structure	TLC hR_f Value*
IV-181A	morpholino	2R,3S	43
IV-206A	morpholino	2R,3R	40
IV-230A	pyrrolidino	2R,3S	31
IV-231B	pyrrolidino	2R,3R	31

* TLC solvent: C-M-HOAc 90:5:10. Similar but faster migrations were obtained with solvent A.

Structure-activity correlations. The results of testing the compounds in an assay system for GlcCer synthase are listed in Table 3. Each inhibition determination (± SD) shown in Table 3 was carried out in triplicate. Some of the inhibitors were tested as mixtures of DL-erythro- and DL-threo-isomers (see column 4). Only the D-threo enantiomer in each mixture was predicted to be the actual enzyme inhibitor (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987)); the content of this isomer was calculated by measuring the proportions of the threo- and erythro- racemic mixtures by quantitative TLC. The DL-threo contents were found to be in the range of 40 to 72%. The comparisons, in the case of the mixtures, are therefore approximate (most of the samples were not purified to remove the three less-active isomers and the observed data were not corrected for the level of the primary enantiomers). The separation of the threo- and erythro- forms is most conveniently accomplished by crystallization, but the specific conditions vary for each substance; thus only BML-119, a strong inhibitor, was seperated into its threo- and erythro- forms. BML-

112 is not included in Table 3 because it had no inhibitory activity against GlcCer synthase of rabbit liver microsomes.

TABLE 3
Inhibition of Ceramide Glucosyltransferase of MDCK cell Homogenates by Different Compounds

Inhbitor Number	% Inhibition at 80 μM	Inhibition at 5 μM	Active Isomer[h]
BML-113	60 ± 4.7[a]		29
BML-114	31 ± 2.9[a]		20
BML-115	84 ± 0.8[a] 82 ± 0.3[b]	12.4 ± 0.7[f]	27
BML-116	28 ± 3.2[a]		27
BML-117	35 ± 0.6[b]		36
BML-118	62 ± 0.4[b]	8.3 ± 1.4[f]	32
BML-119	94 ± 1.4[b] 97 ± 0.1[c] 96 ± 0.1[d]	51 ± 2.3[e] 49 ± 0.8[f]	29
BML-120	11 ± 3.0[c]		26
BML-121	11 ± 0.4[c]		28
BML-122	58 ± 1.6[d]		26
BML-123	86 ± 0.1[d]	15 ± 0.8[f]	33
BML-124	-2 ± 1.6[d]		15
BML-125		9 ± 3.0[e]	26
BML-126	60 ± 1.8[e]	54 ± 0.3[f]	34
PDMP	90 ± 0.8[e]	16 ± 1.8[f]	100
PPMP		32 ± 1.8[e] 32 ± 0.7[f]	100
IV-181A		12 ± 0.2[g]	100
IV-206A		73 ± 1.5[g]	100
IV-230A		19 ± 2.1[g]	100
IV-231B		87 ± 0.4[g]	100

[a-g] Different samples were assayed as parts of different experiments.
[h] Percent of the active D-stereoisomer in the synthesized sample, estimated by scanning the two stained bands, assuming the slower one was the (racemic) active form.

Comparison of PDMP (1R,2R-decanoate) and PPMP (1R,2R-palmitate), when evaluated at the same time in Expt. f, shows that an increase in the chain length of the N-acyl group from 10 to 16 carbon atoms distinctly improved the inhibitory activity against GlcCer synthase, as noted before (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.* 111:191-196 (1992)). Accordingly, most of the other compounds were synthesized with the palmitoyl group for comparison with PPMP. The comparisons between the best inhibitors are clearer at the 5 μM level.

Replacing the oxygen in the morpholine ring of PPMP with a methylene group (BML-115) improved activity ~1.4-fold (calculated from the inhibitions at 5 μM in Expt. f and relative purities, and assuming that the percent inhibition is proportional to concentration in this region: 12.4/27 x 100/32 = 1.4). Previous comparison with mouse brain, human placenta, and human Gaucher spleen glucosyltransferase also showed that replacing the morpholino ring with the piperidino ring in a ketone analog of PDMP (1-phenyl-2-decanoylamino-3-piperidino-1-propanone) produced a much more active inhibitor (Vunnam, R.R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids* 26:265-278 (1980)).

Replacing the piperidine group with a 7-membered ring (BML-116) greatly decreased the activity, while use of a 5-membered ring (BML-119) quadrupled the effectiveness (50 vs 12.4% inhibition). A 4-membered ring (BML-123) yielded a compound about as effective as the piperidino compound. The parent amine (BML-124), its *N,N*-diethyl analog (BML-114), and the sterically bulky *N*-phenylpiperazine analog (BML-112) displayed little or no activity.

Replacing a hydrogen atom with a fluorine atom in the *p*-position of the phenyl ring decreased the inhibitory power (BML-117 vs PDMP and BML-118 vs BML-115). Substitution of the *p*-position with an electron-donating moiety, the methoxy group, had a similar weakening effect in the case of the morpholino compound (BML-125 vs PPMP). Comparison of the pyrrolidino compounds, which are more basic than the morpholino compounds, showed that the methoxy group enhanced the inhibitory power (BML-126 vs BML-119).

Preparations of BML-119 were separated into threo and erythro racemic mixtures by HPLC on a Waters Microbondapak C_{18} column, using M-W-conc. NH_4OH 90:10:0.2 as the elution solvent. The material eluting earlier (but migrating more slowly on a TLC plate) was called BML-130; the later eluting material (faster

by TLC) was called BML-129. Assay of GlcCer synthase with each preparation at 5 μM showed 15% inhibition by BML-129 and 79% inhibition by BML-130. TLC analysis of the two preparations revealed incomplete separation, which could explain the minor inhibition by BML-129. When the two stereoisomers were separated by preparative TLC, the difference in effectiveness was found to be somewhat higher, evidently due to the better separation by this method. Thus the slower-migrating stereoisomer accounted for all or nearly all of the inhibitory activity, as noted with PDMP (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.* 28:565-571 (1987)).

Comparison of the two pairs of aliphatic inhibitors (bottom of Table 3) showed that the 2R,3R (D-threo) form is the primary inhibitor of glucosyltransferase. This finding is in agreement with previous identification of the active PDMP isomer as being the D-threo enantiomer. However, unlike the aromatic analog, BML-129 (2R,3S/2S,3R), there was a relatively small but significant activity in the case of the (erythro) 2R,3S stereoisomer. The erythro form of PDMP was found to inhibit cell proliferation of rabbit skin fibroblasts almost as well as R,R/S,S-PDMP but it did not act on the GSLs (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem. (Tokyo)* 108:525-530 (1990)). As noted with the aromatic analogs, the pyrrolidine ring was more effective than the morpholine ring (Table 3).

Comparison of the aliphatic and corresponding aromatic inhibitors can be made in the case of the optically active morpholine compounds PPMP and IV-206A, both of which have the R,R structure and the same fatty acid. Here it appears that the aliphatic compound is more effective (Table 3). However in a second comparison, at lower concentrations with the inhibitors incorporated into the substrate liposomes, the degree of inhibition was 77 $\pm$ 0.9% with 3 μM IV-231B and 89 $\pm$ 0.6% with 6 μM DL-threo BML-119.

Evaluations of cultured cell growth. Exposure of five different cancer cell lines to inhibitors at different concentrations for 4 or 5 days showed that the six BML compounds most active against GlcCer synthase were very effective growth inhibitors (Table 4). The IC_{50} values (rounded off to one digit in the table) ranged from 0.7 to 2.6 μM.

TABLE 4
Inhibition of Tumor Cell Growth *In Vitro* by Various Inhibitors

Cell Type	BML-115	BML-118	BML-119	BML-123	BML-126	BML-129	BML-130
MCF-7	2	2	2	2	1	3	2
H-460	2	2	1	1	1	2	3
HT-29	2		1	2	1	2	2
9L	2	2	1	2	2	2	2
UMSCC -10A	1		1	1	1	2	2

Figure 1 shows growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors, as described above. The BML compounds were used as synthesized (mixtures of DL-*threo* and *-erythro* stereoisomers) while the PDMP and PPMP were optically resolved R,R isomers. The concentrations shown are for the mixed racemic stereoisomers, since later work (Table 4) showed that both forms were very similar in effectiveness. Figure 1 illustrates the relatively weak effectiveness of R,R-PPMP and even weaker effectiveness of R,R-PDMP. The three new compounds, however, are much better inhibitors of GlcCer synthase and growth. These differences in growth inhibitory power correlate with their effectiveness in MDCK cell homogenates as GlcCer synthase inhibitors. Some differences can be expected due to differences in sensitivity of the synthase occurring in each cell type (the synthases were assayed only in MDCK cells).

Growth inhibition by each of the most active BML compounds occurred in an unusually small range of concentrations (*e.g.*, the slopes of the cytotoxic regions are unusually steep). Similar rapid drop-offs were seen in another series of tests with 9L cells, in which BML-119 yielded 71% of the control growth with 1 μM inhibitor, but only 3% of control growth with 3 μM. Growth was 93% of control growth with 2 μM BML-130 but only 5% of controls with 3 μM inhibitor. While some clinically useful drugs also show a narrow range of effective concentrations, this is a relatively uncommon relationship.

When the erythro- and threo-stereoisomeric forms of BML-119 (-129 and -130) were compared, they were found to have similar effects on tumor cell growth (Table 4). This observation is similar to the results with PDMP isomers in fibroblasts

cited above (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem. (Tokyo)* 108:525-530 (1990)). Since enzymes are optically active and since stereoisomers and enantiomers of drugs can differ greatly in their effect on enzymes, it is likely that BML-129 and BML-130 work on different sites of closely related metabolic steps.

Figure 2 shows the amount of cellular protein per dish for MDCK cells cultured for 24 h in medium containing different concentrations of the separated erythro- and threo- isomers of BML-119, as percent of the incorporation by cells in standard medium. Each point shown in Figure 2 is the average of values from three plates, with error bars corresponding to one standard deviation.

Figure 3 shows [^{3}H]thymidine incorporation into DNA of MDCK cells incubated as in Figure 2. The values in Figure 3 are normalized on the basis of the protein content of the incubation dishes and compared to the incorporation by cells in standard medium.

Figures 2 and 3 thus provide comparison of the two stereoisomers with MDCK cells. The isomers were found to inhibit growth and DNA synthesis with similar effectiveness. Thus the MDCK cells behaved like the human tumor cells with regard to IC_{50} and the narrow range of concentrations resulting in inhibition of protein and DNA synthesis.

Surprisingly, the aliphatic inhibitor IV-231B exerted no inhibitory effect on MDCK cell growth when incubated at 20 μM for 1 day or 1 μM for 3 days. Tests with a longer growth period, 5 days, in 5 μM inhibitor also showed no slowing of growth. The dishes of control cells, which contained BSA as the only additive to the medium, contained 3.31 $\pm$ 0.19 mg of protein, while the IV-231B/BSA treated cells contained 3.30 $\pm$ 0.04 mg.

Lipid changes induced in the cells. Examination by TLC of the alkali-stable MDCK lipids after a 24 h incubation disclosed that BML-130 was more effective than BML-129 in lowering GlcCer levels, as expected from its greater effectiveness in vitro as a glucosyltransferase inhibitor. The level of GlcCer, estimated visually, was greatly lowered by 0.3 μM BML-130 or 0.5 μM BML-129. The levels of the other lipids visible on the plate (mainly sphingomyelin (SM), cholesterol, and fatty acids) were changed little or not at all. BML-129 and the GlcCer synthase inhibitor, BML-130, were readily detected by TLC at the various levels used, showing that they were taken up by the cells during the incubation period at dose-dependent rates. Lactosylceramide overlapped the inhibitor bands

with solvent D but was well separated with solvent E, which brought the inhibitors well above lactosylceramide.

Ceramide accumulation was similar for both stereoisomers (data not shown). An unexpected finding is that noticeable ceramide accumulation appeared only at inhibitor concentrations that were more than enough to bring GlcCer levels to a very low point (*e.g.*, at 2 or 4 μM). The changes in ceramide concentration were quantitated in a separate experiment by the diglyceride kinase method, which allows one to also determine diacylglycerol (DAG) concentration (Preiss, J.E. et al., "Quantitative Measurement of SN-1,2-Diacylglycerols Present in Platelets, Hepatocytes, and Ras- and Sis-Transformed Normal Rat Kidney Cells," *J. Biol. Chem.* 261:8597-8600 (1986)). The results (Table 5) are similar to the visually estimated ones: at 0.4 μM BML-129 or -130 there was little effect on ceramide content but at 4 μM inhibitor, a substantial increase was observed. (While the duplicate protein contents per incubation dish were somewhat erratic in the high-dose dishes, in which growth was slow, the changes were nevertheless large and clear.) Accumulation of ceramide had previously been observed with PDMP, at a somewhat higher level of inhibitor in the medium (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). From the data for cellular protein per incubation dish, it can be seen that there was no growth inhibition at the 0.4 μM level with either compound but substantial inhibition at the 4 μM level, especially with the glucosyltransferase inhibitor, BML-130. This finding is similar to the ones made in longer incubations with human cancer cells.

TABLE 5
Effects of BML-129 and -130 on MDCK Cell Growth and the Content of Ceramide and Diacylglycerol

Growth Medium	Protein	Ceramide	Diglyceride
	μg/dish	nmol/mg protein	
Controls	490 560	1.04 0.96	4.52 5.61
0.4 μm BML-129	500 538	1.29 0.99	5.51 5.13
0.4 μm BML-130	544 538	0.94 0.87	4.73 5.65
4 μm BML-129	396 311	3.57 3.78	9.30 9.68
4 μm BML-130	160 268	5.41 3.34	11.9 8.71

In a separate study of ceramide levels in MDCK cells, BML-130 at various concentrations was incubated with the cells for 24 h. The ceramide concentration, measured by TLC densitometry, was 1.0 nmol/mg protein at 0.5 μM, 1.1 at 1 μM, 1.5 at 2 μM, and 3.3 at 4 μM. The results with BML-129 were virtually identical.

It is interesting that the accumulation of ceramide paralleled an accumulation of diacylglycerol (DAG), as observed before with PDMP (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). DAG is ordinarily considered to be an activator of protein kinase C and thus a growth stimulator, but the low level of GlcCer in the inhibited cells may counteract the stimulatory effect. Ceramide reacts with lecithin to form SM and DAG, so it is possible that the increased level of the latter reflects enhanced synthesis of the phosphosphingolipid rather than an elevated attack on lecithin by phospholipase D. Arabinofuranosylcytosine (ara-C), an antitumor agent, also produces an elevation in the DAG and ceramide of HL-60 cells (Strum, J.C. et al., "1-β-D-Arabinofuranosylcytosine Stimulates Ceramide and Diglyceride Formation in HL-60 Cells," *J. Biol. Chem.* 269:15493-15497 (1994)).

TLC of MDCK cells grown in the presence of 0.02 to 1 μM IV-231B for 3 days showed that the inhibitor indeed penetrated the cells and that there was a great depletion of GlcCer, but no ceramide accumulation. The depletion of GlcCer was

evident even at the 0.1 μM level and virtually no GlcCer was visible at the 1 μM level; however the more polar GSLs were not affected as strongly. After incubation for 5 days in 5 μM inhibitor, all the GSLs were virtually undetectable. The ceramide concentrations in the control and depleted cells were very similar: 13.5 ± 1.4 vs 13.9 ± 0.2 μg/mg protein.

The lack of ceramide accumulation in cells exposed to the aliphatic inhibitors was examined further to see if it might be due to differential actions of the different inhibitors on additional enzymes involving ceramide metabolism. For example, IV-231B might block ceramide synthase and thus prevent accumulation despite the inability of the cells to utilize ceramide for GlcCer synthesis. However, assay of ceramide synthase in homogenized cells showed it was not significantly affected by 5 μM inhibitors (Table 6). There did appear to be moderate inhibition at the 50 μM level with PDMP and the aliphatic inhibitor.

TABLE 6
Effect of Inhibitors on Acid and Neutrual Ceramidases and Ceramide Synthase of MDCK Cells

Inhibitor Tested	Enzyme Activity (% of control)		
	Ceramidase pH 4.5	Ceramidase pH 7.4	Ceramide Synthase
D-*threo*-PDMP, 5 μM	97 ± 4	116 ± 19	99 ± 5
D-*threo*-PDMP, 50 μM	133 ± 13*	105 ± 11	66 ± 9*
BML-129, 5 μM	108 ± 8	100 ± 0	97 ± 0
BML-129, 50 μM	171 ± 26*	99 ± 2	102 ± 1
BML-130, 5 μM	107 ± 11	100 ± 15	108 ± 10
BML-130, 50 μM	160 ± 21*	100 ± 15	106 ± 29
IV-231B, 5 μM	106 ± 3	116 ± 20	90 ± 8
IV-231B, 50 μM	113 ± 8	112 ± 3	71 ± 18*

*Notable differences.

Assay of the two kinds of ceramidase (Table 6) showed that there was no effect of either the aliphatic or aromatic inhibitors at the 5 μM level, at which point cell growth is completely stopped in the case of the pyrrolidino compounds. At the 50 μM level, however, the acid enzyme was stimulated markedly by the aromatic inhibitors, particularly the two stereoisomeric forms of the pyrrolidino compound.

Sphingomyelin synthase was unaffected by PDMP or the aliphatic inhibitor but BML-129 and -130 produced appreciable inhibition at 50 μM (54% and 61%, respectively) (Table 7).

TABLE 7
Effect of Inhibitors on Acid and Neutrual Sphingomyelinases and Sphingomyelin Synthase

Inhibitor Tested	Enzyme Activity (% of control) Sphingomyelinase pH 4.5	Sphingomyelinase pH 7.1	Sphingomyelinase Synthase[a]
D-*threo*-PDMP, 5 μM	102 ± 3	121 ± 13	
D-*threo*-PDMP, 50 μM	100 ± 3	108 ± 8	
BML-129, 5 μM	108 ± 4	105 ± 11	84 ± 27
BML-129, 50 μM	97 ± 3	142 ± 11[b]	46 ± 11[b]
BML-130, 5 μM	109 ± 1	110 ± 7	87 ± 14
BML-130, 50 μM	114 ± 2	152 ± 14[b]	39 ± 18[b]
IV-231B, 5 μM	101 ± 7	131 ± 3[b]	
IV-231B, 50 μM	112 ± 11	120 ± 3[b]	

[a] Data for PDMP and IV-231B are not shown here as they were tested in other experiments; no effect was seen.
[b] Notable differences.

Neutral sphingomyelinase (SMase) was distinctly stimulated by the aliphatic inhibitor, IV-231B, even at 5 μM (Table 7). From this one would expect that the inhibitor would produce accumulation of ceramide, yet it did not. The two pyrrolidino compounds produced appreciable stimulation at the 50 μM level. No significant effects were obtained with acid SMase.

Discussion

The present invention shows that the nature and size of the tertiary amine on ceramide-like compounds exerts a strong influence on GlcCer synthase inhibition, a 5-membered ring being most active. It also shows that the phenyl ring used previously to simulate the trans-alkenyl chain corresponding to that of sphingosine could, with benefit, be replaced with the natural alkenyl chain.

Findings with the most active GlcCer synthase inhibitors in growth tests compare favorably with evaluations of some clinically useful chemotherapeutic

agents on three of the tumor cell lines in the same Drug Evaluation Core Laboratory. The IC_{50} values were 0.2 to 6 μM for cisplatin, 0.02 to 44 μM for carboplatin, 0.03 to 0.2 μM for methotrexate, 0.07 to 0.2 μM for fluorouracil, and 0.1 to 1 μM for etoposide. Unlike these agents, the compounds of the present invention yielded rather similar effects with all the cell types, including MDCK cells, and thus have wider potential chemotherapeutic utility. This uniformity of action is consistent with the idea that GSLs play a wide and consistent role in cell growth and differentiation.

An important observation from the MDCK cell study is that strong inhibition of cell growth and DNA synthesis occurred only at the same concentrations of aromatic inhibitor that produced marked ceramide accumulation. This observation supports the assertion that ceramide inhibits growth and enhances differentiation or cell death (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters* 307:211-214 (1992)). It also agrees with previous work with octanoyl sphingosine, a short chain ceramide that produced greatly elevated levels of natural ceramide and slowed growth (Abe, A. et al., "Metabolic Effects of Short-Chain Ceramide and Glucosylceramide on Sphingolipids and Protein Kinase C," *Eur. J. Biochem.* 210:765-773 (1992)). It is also in agreement with a finding that some synthetic, nonionic ceramide-like compounds did not inhibit GlcCer synthase even though they behave like ceramide in blocking growth (Bielawska, A. et al., "Ceramide-Mediated Biology. Determination of Structural and Stereospecific Requirements Through the Use of N-Acyl-Phenylaminoalcohol Analogs," *J. Biol. Chem.* 267:18493-18497 (1992)). Compounds tested included 20 μM D-erythro-N-myristoyl-2-amino-1-phenyl-1-propanol, its L-enantiomer, the four stereoisomers of *N*-acetylsphinganine, and *N*-acetylsphingosine. Furthermore, the lack of growth inhibition and ceramide accumulation in cells treated with the aliphatic inhibitor IV-231B is also consistent with the correlation between ceramide level and growth rate.

The accumulation of ceramide that occurred at higher levels of GlcCer synthase inhibitors could be attributed not only to blockage of ceramide utilization, but also to blockage of SM synthesis or ceramide hydrolase. This possibility is especially relevant to the R,S-, S,R-, and S,S-isomers, which seem to exert effects on sphingolipids without strongly inhibiting GlcCer synthesis. The tests with both the DL-erythro-pyrrolidino inhibitor (BML-129) and the DL-threo-pyrrolidino inhibitor (BML-130), at a level producing strong growth inhibition, showed that neither material at a low concentation inhibited the enzymes tested *in vitro* (Tables 6 and

7) but they did cause growth inhibition as well as accumulation of ceramide. PDMP, at relatively high concentrations (50 μM), was found to inhibit SM synthase in growing CHO cells (Rosenwald, A.G. et al., "Effects of a Sphingolipid Synthesis Inhibitor on Membrane Transport Through the Secretory Pathway," *Biochemistry* 31:3581-3590 (1992)). In the test with MDCK homogenates, it did not inhibit this synthase, in agreement with the finding that labeled palmitate incorporation into SM was stimulated by PDMP (Shayman, J.A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)).

Retinoic acid is a growth inhibitor of interest in cancer chemotherapy and a possible adjunct in the use of the inhibitors of the present invention. It has been found to elevate ceramide and DAG levels (Kalén, A. et al., "Elevated Ceramide Levels in GH4C1 Cells Treated with Retinoic Acid," *Biochim. Biophys. Acta* 1125:90-96 (1992)) and possibly lower lecithin content (Tang, W. et al., "Phorbol Ester Inhibits 13-Cis-Retinoic Acid-Induced Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate in Cultured Murine Keratinocytes: a Possible Negative Feedback Via Protein Kinase C-Activation," *Cell Bioch. Funct.* 9:183-191 (1991)).

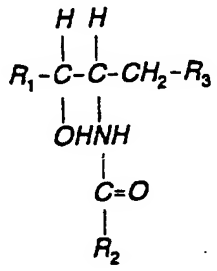
D-threo-PDMP was found to be rather active in delaying tumor cell growth or in producing complete cures in mice (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.* 38:23-30 (1987)) but high doses were needed. From the data in Figure 1, the inhibitors of the present invention are approximately 30 times as active, so the dosage levels are typical of clinically useful drugs. The need to use high doses with PDMP was attributed to rapid inactivation by cytochrome P450 (Shukla, A. et al., "Metabolism of D-[^{3}H]PDMP, an Inhibitor of Glucosylceramide Synthesis, and the Synergistic Action of an Inhibitor of Microsomal Monooxygenase," *J. Lipid Res.* 32:713-722 (1991)). Cytochrome P450 can be readily blocked by various nontoxic drugs such as cimetidine, therefore high levels of the compounds of the present invention can be maintained.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

All publications cited herein are expressly incorporated by reference.

WE CLAIM:

1. A method for inhibiting the growth of cancer cells in a mammal comprising the step of administering to the mammal a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 10 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

2. The method of Claim 1, where the growth of the cancer cells is inhibited by increasing ceramide levels in the cancer cells to a toxic level.

3. The method of Claim 1, where R_1 is a phenyl group.

4. The method of Claim 1, where R_1 is *p*-methoxyphenyl.

5. The method of Claim 1, where R_3 is pyrrolidino.

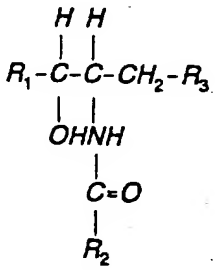
6. The method of Claim 1, where R_2 is $C_{15}H_{31}$.

7. The method of Claim 6, where R_1 is $CH=CH(CH_2)_{12}CH_3$.

8. The method of Claim 1, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

9. The method of Claim 1, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

10. A method for treating a patient having sphingolipidosis by reducing glycosphingolipid synthesis comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

11. The method of Claim 10, where R_1 is a phenyl group.

12. The method of Claim 10, where R_1 is *p*-methoxyphenyl.

13. The method of Claim 10, where R_3 is pyrrolidino.

14. The method of Claim 10, where R_2 is $C_{15}H_{31}$.

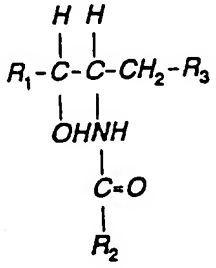
15. The method of Claim 10, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

16. The method of Claim 10, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

17. The method of Claim 10, where the patient is diagnosed as having Gaucher disease.

18. The method of Claim 10, where the patient is diagnosed as having Tay-Sachs disease.

19. A method for decreasing excessive cell growth or cell division in a mammal comprising the step of administering to the mammal a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

20. The method of Claim 19, where R_1 is a phenyl group.

21. The method of Claim 19, where R_1 is *p*-methoxyphenyl.

22. The method of Claim 19, where R_3 is pyrrolidino.

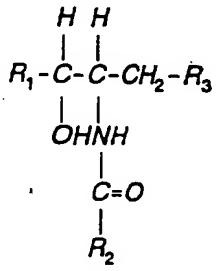
23. The method of Claim 19, where R_2 is $C_{15}H_{31}$.

24. The method of Claim 19, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

25. The method of Claim 19, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

26. The method of Claim 19, where the cell growth or cell division is of the smooth muscle cells of the mammal's arteries.

27. A method for treating a patient having a microbial or viral infection comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

28. The method of Claim 27, where R_1 is a phenyl group.

29. The method of Claim 27, where R_1 is *p*-methoxyphenyl.

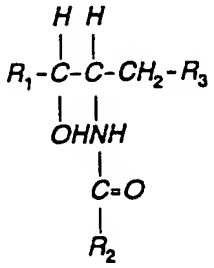
30. The method of Claim 27, where R_3 is pyrrolidino.

31. The method of Claim 27, where R_2 is $C_{15}H_{31}$.

32. The method of Claim 27, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

33. The method of Claim 27, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

34. A method for treating a patient having a drug resistant tumor comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

35. The method of Claim 34, where R_1 is a phenyl group.

36. The method of Claim 34, where R_1 is *p*-methoxyphenyl.

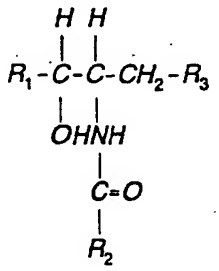
37. The method of Claim 34, where R_3 is pyrrolidino.

38. The method of Claim 34, where R_2 is $C_{15}H_{31}$.

39. The method of Claim 34, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

40. The method of Claim 34, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

41. A method for reducing tumor angiogenesis in a patient comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

42. The method of Claim 41, where R_1 is a phenyl group.

43. The method of Claim 41, where R_1 is *p*-methoxyphenyl.

44. The method of Claim 41, where R_3 is pyrrolidino.

45. The method of Claim 41, where R_2 is $C_{15}H_{31}$.

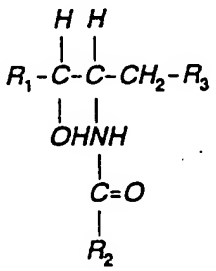
46. The method of Claim 41, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

47. The method of Claim 41, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

48. A vaccination method comprising the steps of:

a) removing cancer cells from a patient;

b) treating the cancer cells *in vitro* with an effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof; and

c) administering the treated cells to the patient.

49. The method of Claim 48, where R_1 is a phenyl group.

50. The method of Claim 48, where R_1 is *p*-methoxyphenyl.

51. The method of Claim 48, where R_3 is pyrrolidino.

52. The method of Claim 48, where R_2 is $C_{15}H_{31}$.

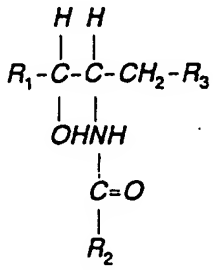
53. The method of Claim 48, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

54. The method of Claim 48, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

AMENDED CLAIMS

[received by the International Bureau on 24 February 1997 (24.02.97); original claims 1-54 replaced by amended claims 1-45 (7 pages)]

1. A method for treating a patient having sphingolipidosis by reducing glycosphingolipid synthesis comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

2. The method of Claim 1, where R_1 is a phenyl group.

3. The method of Claim 1, where R_1 is *p*-methoxyphenyl.

4. The method of Claim 1, where R_3 is pyrrolidino.

5. The method of Claim 1, where R_2 is $C_{15}H_{31}$.

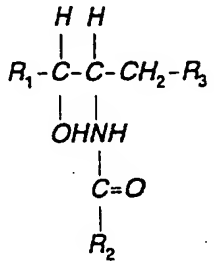
6. The method of Claim 1, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

7. The method of Claim 1, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

8. The method of Claim 1, where the patient is diagnosed as having Gaucher disease.

9. The method of Claim 1, where the patient is diagnosed as having Tay-Sachs disease.

10. A method for decreasing excessive cell growth or cell division in a mammal comprising the step of administering to the mammal a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

11. The method of Claim 10, where R_1 is a phenyl group.

12. The method of Claim 10, where R_1 is *p*-methoxyphenyl.

13. The method of Claim 10, where R_3 is pyrrolidino.

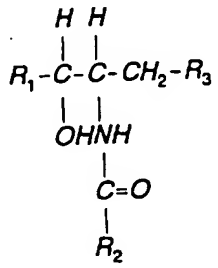
14. The method of Claim 10, where R_2 is $C_{15}H_{31}$.

15. The method of Claim 10, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

16. The method of Claim 10, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

17. The method of Claim 10, where the cell growth or cell division is of the smooth muscle cells of the mammal's arteries.

18. A method for treating a patient having a microbial or viral infectioncomprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

19. The method of Claim 18, where R_1 is a phenyl group.

20. The method of Claim 18, where R_1 is *p*-methoxyphenyl.

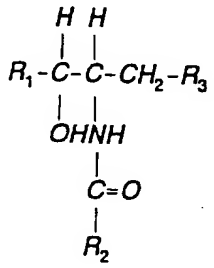
21. The method of Claim 18, where R_3 is pyrrolidino.

22. The method of Claim 18, where R_2 is $C_{15}H_{31}$.

23. The method of Claim 18, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

24. The method of Claim 18, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

25. A method for treating a patient having a drug resistant tumor comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

26. The method of Claim 25, where R_1 is a phenyl group.

27. The method of Claim 25, where R_1 is *p*-methoxyphenyl.

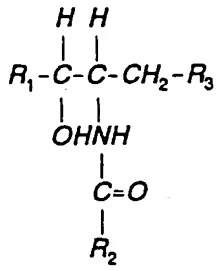
28. The method of Claim 25, where R_3 is pyrrolidino.

29. The method of Claim 25, where R_2 is $C_{15}H_{31}$.

30. The method of Claim 25, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

31. The method of Claim 25, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

32. A method for reducing tumor angiogenesis in a patient comprising the step of administering to the patient a therapeutically effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof.

33. The method of Claim 32, where R_1 is a phenyl group.

34. The method of Claim 32, where R_1 is *p*-methoxyphenyl.

35. The method of Claim 32, where R_3 is pyrrolidino.

36. The method of Claim 32, where R_2 is $C_{15}H_{31}$.

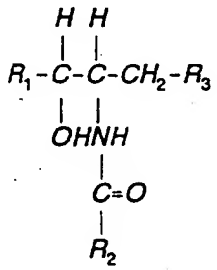
37. The method of Claim 32, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

38. The method of Claim 32, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

39. A vaccination method comprising the steps of:

a) removing cancer cells from a patient;

b) treating the cancer cells *in vitro* with an effective amount of a compound selected from the group consisting of compounds of the formula:



where R_1 is an aromatic structure, an alicyclic structure, a branched aliphatic structure or a linear aliphatic group having 5 to 15 carbons;

R_2 is an aliphatic chain having 9 to 18 carbons; and

R_3 is an amine group;

and functional homologues, isomers and pharmaceutically acceptable salts thereof; and

c) administering the treated cells to the patient.

40. The method of Claim 39, where R_1 is a phenyl group.

41. The method of Claim 39, where R_1 is *p*-methoxyphenyl.

42. The method of Claim 39, where R_3 is pyrrolidino.

43. The method of Claim 39, where R_2 is $C_{15}H_{31}$.

44. The method of Claim 39, where the compound is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol and pharmaceutically acceptable salts thereof.

45. The method of Claim 39, where the compound is 1-pyrrolidino-1-deoxyceramide and pharmaceutically acceptable salts thereof.

Figure 1

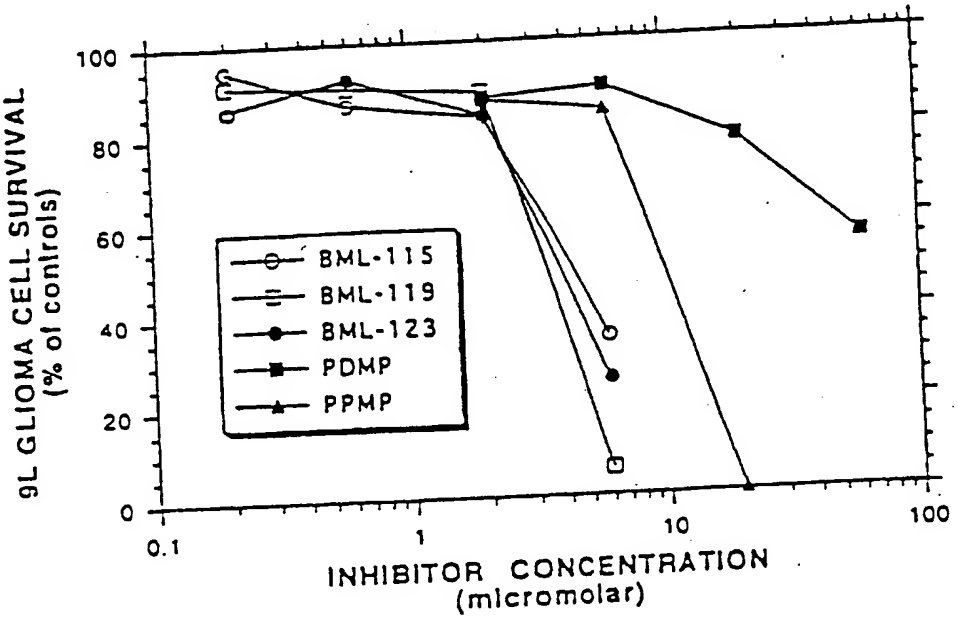


Figure 2

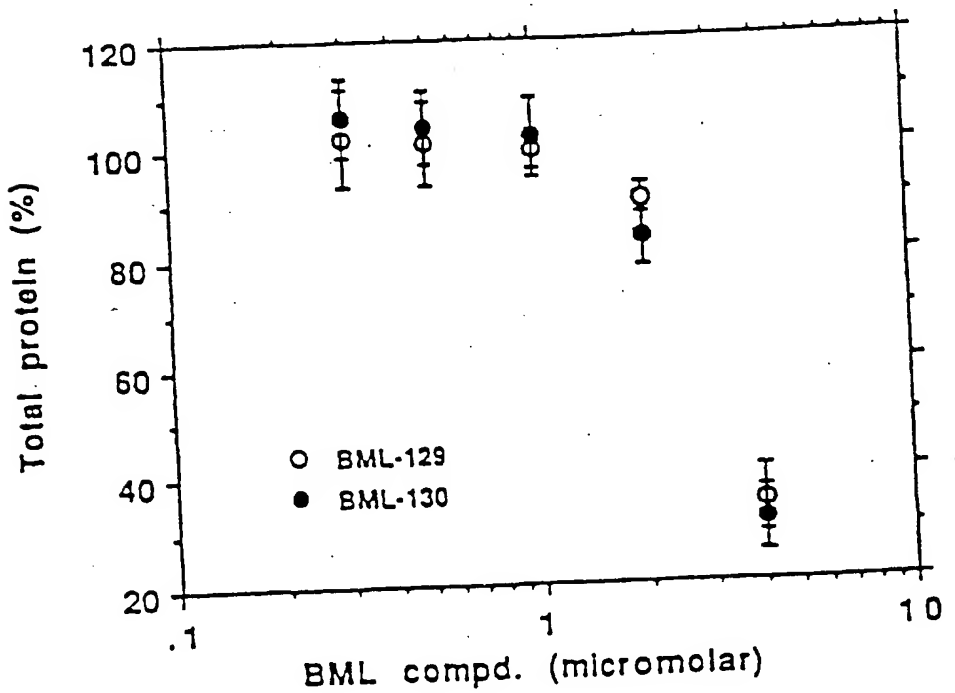
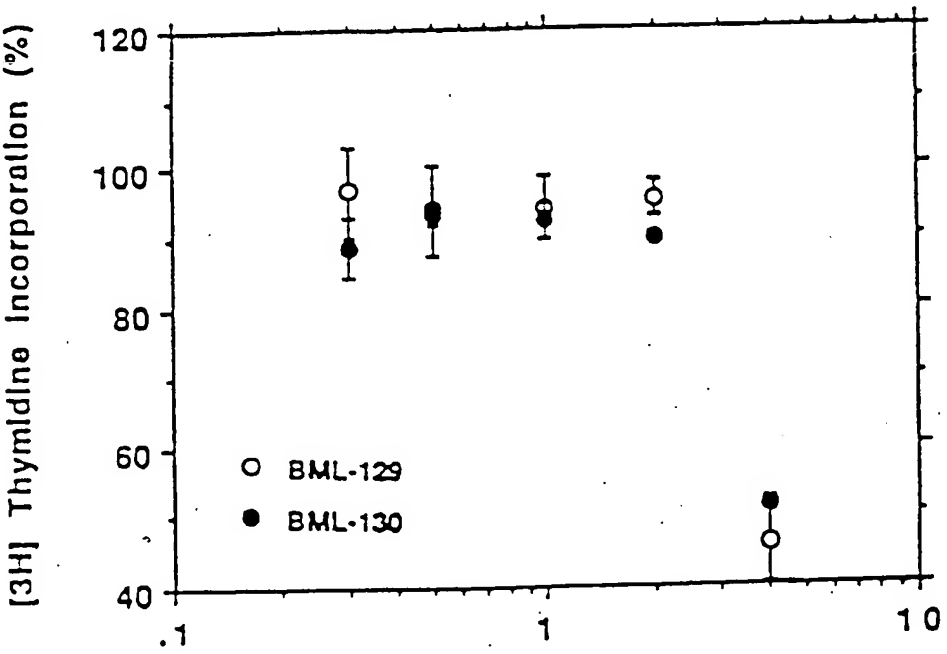


Figure 3


[3H] Thymidine Incorporation (%)
120
100
80
60
40
.1
1
10
BML-129
BML-130

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14219

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/16, 31/40
US CL :514/428, 625
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/428, 625

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE, structure plus use.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chem. abstr., Vol. 122, No. 23, 05 June 1995 (Columbus. OH, USA), page 32, abstract 281432s, ABE et al. 'Structural and Stereochemical Studies of Potent Inhibitors of Glucosylceramide Synthase and Tumor Cell Growth,' J. Lipid Res., 1995 36(3), 611-621, see entire abstract.	1-54

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 DECEMBER 1996	Date of mailing of the international search report 24 DEC 1996
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Form PCT/ISA/210 (second sheet)(July 1992)*